Molecular Characterization of *Sugarcane Yellow Leaf Virus* (SCYLV) and its Effect on Sucrose Transporters in Sugarcane *Saccharum spp.* hybrids

DISSERTATION

zur Erlangung des akademischen Grades eines
Doktor der Naturwissenschaften (Dr. rer. nat.)
im Fach Biologie der Fakultät für Biologie, Chemie und Geowissenschaften
der Universität Bayreuth

vorgelegt von

M.Sc. Abdelaleim Ismail Ibrahim ElSayed

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Die vorliegende Arbeit wurde in der Zeit von Oktober 2006 bis Juli 2010 am Lehrstuhl Pflanzen Physiologie / der Universität Bayreuth unter der Betreuung von Herrn Prof. Dr. Ewald Komor angefertigt.

Vollständiger Abdruck der von der Fakultät für Biologie, Chemie und Geowissenschaften der Universität Bayreuth genehmigten Dissertation zur Erlangung des akademischen Grades Doktor der Naturwissenschaften (Dr. rer. nat.).

Amtierender Dekan:	Prof. Dr. Stephan Clemens
Tag des Einreichens der Dissertation:	26.05.2010
Tag des wissenschaftlichen Kolloquiums:	15.07.2010

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Freedom is not worth having if it does not include the freedom to make mistakes.

Dedication

To my father, my mother and my brothers and sisters who give me unconditional support and encouragement during all the steps of my life.

To my wife Faten

To my beloved son Mohamed who's crying always gives me a reason to wake up early and stay in the lab as much as possible to work

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1. Summary

Sugarcane is an important crop plant and has served as a source of sugar for hundreds of years, recently it is used to produce bioethanol, a renewable bio-fuel energy source. *Sugarcane yellow leaf virus* (SCYLV) was detected in the late 1990s first in Hawaii as a causal agent of a sugarcane disease (Yellow leaf) which leads to sugarcane yellow leaf syndrome and reduced sugar yield.

The presence of *Sugarcane yellow leaf virus* was determined by RT-PCR in several sugarcane cultivars, mostly from Hawaii. Interesting was the comparison of so-called susceptible versus resistant cultivars. As expected, the susceptible Hawaiian cultivars H73-6110 and H87-4094 showed strong PCR amplification products of SCYLV, while the virus-free line H87-4094, produced by tissue culture, showed no PCR product. The three resistant cultivars H87-4319, H78-4153 and H78-7750 showed quite different amplification patterns. While H78-4153 and H78-7750 expressed a weak but specific band of the correct size, unexpectedly H87-4319 showed strong amplification product. Three Cuban cultivars (C1051-73, JA-605 and CP52-43) showed low titer of SCYLV. No PCR amplificate was obtained with the moderately susceptible cultivar H65-7052. Aphids feeding on cv. H87-4094 contained sufficient virus to yield a SCYLV-signal similar in strength as from preparations from resistant cultivars. Northern blot analysis supported the results obtained from RT-PCR. The presence of SCYLV in the cultivars with low amount of virus titer (H87-4319, H78-7750 and H78-4153) indicated that they should better be called tolerant for the virus in the sense that they allow a low replication rate for SCYLV.

Northern blots showed that RNA of SCYLV is divided into genomic RNA (gRNA) and two subgenomic RNAs (sgRNAs). The estimated molecular size of the gRNA is 6.0 kb, the estimated sizes of the sgRNAs are 1.0 and 2.4 kb. It is known that plant RNA viruses have evolved numerous strategies for genome expression to invade host plants, such as divided genomes, subgenomic messenger RNAs, overlapping reading frames or stop codon suppression. Virus preparations from 3 Hawaiian cultivars (two susceptible and one resistant) were fully sequenced. Quantitative analysis for four different genome regions of SCYLV covering the 6 ORFs has been performed for these 3 cultivars using the GeXP analysis system. The transcript levels of the different regions of SCYLV in these cultivars were present at very different quantities, for example ORF0-1 transcripts were up to 10 times more frequent than transcripts of ORF3-4.

The SCYLV-sequences from the 3 Hawaiian cultivars were aligned to published full and partial sequences. The phylograms corroborated previous findings that the so-called YLS-segment coding for the coat protein shows the least genetic diversity, whereas the other sequence

fragments A-D, representing the ORFs 0-5, expressed a twofold higher diversity. The phylograms of partial sequences and of the whole genome placed the Hawaiian SCYLV-strains next to the Peru strain, apart from the BRA-strains and well apart from the REU-strains. It is proposed that the Hawaiian SCYLV is considered as own group together with the Peru strain as HAW-PER. The sequences from the two susceptible cultivars had a deletion of 48 to 54 nt in ORF1, which codes for the gene silencing suppressor/RNA-dependent RNA-polymerase complex. It is speculated that this deletion is important for the proliferation rate of the virus in the plant.

Sucrose is the main product of sugarcane, which accumulates in the stalk internodes in excess of 50 % of the dry weight. To gain an overview of the physiological status of SCYLV-infected sugarcane compared to virus-free plants, gene expression, transcript levels of sucrose transporter and sugar contents were measured. Sucrose increased rapidly between internodes 3 and 7, reaching a maximum in internodes 7. Sugars content in leaves, seedling and internodes were increased as effect of the SCYLV-infection. Sucrose phosphate synthase (SPSII) transcript levels were approximately the same in sink, source and internodes with a trend to be higher in the mature internodes. A sucrose transporter of Hawaiian cultivar was isolated and sequenced and classified as ShSUT1A. There is high variability among the SUT1 subfamily with identities of 70-97%. The identity between ShSUT1A and ShSUT1 was 97.4%. It is expressed in sink, source and storage tissues. The ShSUT1A was expressed at approximately similar extent in SCYLV-infected and virus-free sugarcane. In addition a partial sequence of a sucrose transporter belonging to the SUT4 family was first obtained in sugarcane and its transcript levels in plant organs were measured. Quantitative analysis for sucrose transporters (ShSUT1 and ShSUT4) using the GeXP analysis system showed that sucrose transporter ShSUT1 was at a higher transcript expression than ShSUT4 in sink and source leaves, but not in mature internodes.

In conclusion,

- SCYLV from Hawaiian cultivars was characterized as belonging to an own subgroup (HAW-PER),
- A deletion of 48-54 nt was detected in the SCYLV-sequence from susceptible cultivars, which may be correlated to virus proliferation, and
- large differences in transcript levels of the viral ORFs were found.
- Sucrose transporter transcripts and SPSII transcripts were not strictly correlated to SCYLV-infection and do not explain the pathological effect of SCYLV on sugarcane.

Zusammenfassung

Zuckerrohr ist eine wichtige Weltwirtschaftspflanze, die seit Jahrhunderten als Zuckerquelle und neuerdings als nachwachsende Energiequelle z. B. für Bio-Ethanol dient. In den 1990ern wurde Zuckerrohr-Gelbblatt-Virus (*Sugarcane yellow leaf virus*, SCYLV) als Ursache für die Gelbblatterkrankung von Zuckerrohr und der daraus erfolgten Ernteminderung entdeckt.

SCYLV wurde mittels RT-PCR in mehreren Zuckerrohrkultivaren, die meisten davon aus Hawaii, nachgewiesen. Interessant war der Vergleich von sogenannten suszeptiblen und resistenten Kultivaren. Erwartungsgemäß ergaben die suszeptiblen Kultivare H73-6110 und H87-4094 mächtige PCR-Banden für SCYLV, während die virusfreie Linie von H87-4094, die aus Gewebekultur gewonnen worden war, kein Amplifikat zeigte. Die 3 resistenten Kultivare zeigten unterschiedliche Ergebnisse. Während H78-4153 und H78-7750 nur schwache Banden erzeugten, wurde bei H87-4319 unerwarteterweise eine starke Amplifikation beobachtet. Drei cubanische Kultivare (C1051-73, JA-605, CP52-43) zeigten einen niedrigen SCYLV-Titer. Das gemäßigt suszeptible Kultivar H65-7052 erbrachte kein SCYLV-Amplifikat. Aphiden, die von infiziertem H87-4094 entnommen wurden, ergaben ein Amplifikat in ähnlicher Stärke wie die resistenten Zuckerrohrkultivare. Ergebnisse von Northern Blots unterstützten die Befunde aus RT-PCR. Wegen der Tatsache, dass die resistenten Kultivare SCYLV, wenn auch in niedrigem Titer, enthielten, sollten sie besser als virus-tolerant bezeichnet werden.

Die Northern Blots zeigten, dass die RNA von SCYLV als gesamtes Genom von 6,0 kb und als (mindestens) 2 subgenomische Fragmente von 1,0 und 2,4 kb vorliegt. Es ist bekannt, dass Pflanzenviren mehrere genetische Strategien entwickelt haben um ihre Wirte zu besiedeln, z. B. geteilte Genome, subgenomische RNAs, überlappende *reading frames* oder *stop-codon*-Unterdrückung. Viruspräparationen aus 3 hawaiianischen Kultivaren (2 suszeptible und 1 resistentes) wurden sequenziert. Die Menge viraler Transkripte von 4 Fragmenten, die die 6 ORFs abdeckten, wurde mittels GEXP in den 3 Kultivaren bestimmt. Die Transkripte dieser SCYLV-Abschnitte waren zu sehr unterschiedlichem Ausmaß vorhanden, beispielsweise war das Fragment zu ORF0-1 bis zu 10fach mehr vorhanden als das Fragment zu ORF3-4.

Die SCYLV-Sequenzen der 3 hawaiianischen Kultivare wurden mit publizierten Sequenzen verglichen und phylogenetisch analysiert. Das sogenannte YLS-Segment zeigte sich als das konservierteste, während die anderen Segmente eine doppelt so hohe Diversität zeigten. Das Phylogram platzierte den hawaiianischen SCYLV-Stamm zusammen mit einem Stamm aus Peru als separate Gruppe, genannt HAW-PER, abgetrennt von BRA-Stämmen und REU-Stämmen. Die viralen Sequenzen aus den beiden suszeptiblen Kultivaren hatten eine 48-54 nt lange Deletion in ORF1, welcher für ein *gene silencing*/RNA-abhängige RNA-Polymerase-Komplex

codiert. Es wird spekuliert, dass diese Deletion für die virale Vermehrung in der Pflanze wichtig sein könnte.

Saccharose ist das hauptsächliche Speicherprodukt von Zuckerrohr und kann im Stamm über 50% des Trockengewichts ausmachen. Um den physiologischen Status der SCYLV-infizierten versus virusfreien Pflanze zu erkunden wurden Zuckergehalt und Transkriptmenge für Saccharosetransporter, Saccharose-Phosphat-Synthase II (SPSII) und die viralen Segmente gemessen. Der Saccharosegehalt nahm von Internodium 3 zu 7 stark zu. SCYLV-Infektion erhöhte den Zuckergehalt leicht in Blättern und Internodien. Die Transkriptmengen von SPSII waren etwa gleich hoch in infizierten und virusfreien Pflanzen, mit einer leichten Erhöhung in reifen Internodien. Ein Saccharosetransporter wurde aus einem hawaiianischem Kultivar isoliert und als ShSUT1A klassifiziert. Die Variabilität zwischen den SUT1-Mitgliedern liegt bei 70-97% Identität, ShSUT1 und ShSUT1A sind zu 97,4% identisch. ShSUT1 ist in *sink*, *source* und Internodien exprimiert und findet sich etwa gleich stark in infizierten und virusfreien Pflanzen. Ferner wurde eine Teilsequenz eines weiteren Saccharosetransporters in Zuckerrohr entdeckt, welcher zur SUT4-Gruppe gehört. Die quantitative Transkriptanalyse mittels GEXP zeigte dass ShSUT1 in sink und source Blättern deutlich stärker exprimiert ist als ShSUT4, nicht aber so in reifen Internodien.

Die Ergebnisse können so zusammen gefasst werden:

- SCYLV aus hawaiianischen Zuckerrohrkultivaren gehört zu einer eigenen Gruppe (HAW-PER),
- suszeptible Kultivare enthalten SCYLV mit einer 48-54 nt Deletion, welche mit der Virusvermehrung in Zusammenhang stehen könnte, und
- es gibt große Unterschiede in der Transkription der viralen Genomteile.
- Saccharosetransporter-Transkripte und SPSII-Transkripte waren nicht deutlich unterschieden zwischen infizierten und virusfreien Pflanzen und können deshalb nicht als kausale Erklärung der SCYLV-Symptome dienen.

2. Introduction

Sugarcane (*Saccharum spp.)* is an important tropical and subtropical crop and served as a source of sugar for centuries. Sugarcane belongs to the grass family (Poaceae), an economically important seed plant family that includes cereals such as maize, wheat, rice, and sorghum as well as many forage crops. The commercial sugarcane cultivars are interspecific hybrids that, under ideal conditions, are capable of storing sucrose in the parenchyma tissues of the stem up to 60% of the dry weight (Moore, 1995). It is generally used to produce sugar and has recently gained increased attention because ethanol derived from cane sugar represents an important renewable bio-fuel energy source, which could turn it into global commodity and important energy source. So far only the fibrous residual of sugar extraction, the so-called bagasse, is already used for electricity generation and is providing surplus electricity in some tropical countries. There is increased interest in this crop due to the impending need to decrease fossil fuel usage.

a. *Sugarcane yellow leaf virus*

There are several sugarcane diseases caused by bacteria, fungi and viruses. Concerning the viral diseases, there are approximately seven viruses of international importance in sugarcane production: *Sugarcane mosaic virus* (SCMV), *Sugarcane streak virus* (SSC), *Peanut clump virus* (PCV), *Sugarcane bacilliform virus* (SCBV), *Sugarcane mild mosaic virus* (SCMMV), *Fiji disease virus* (FDV) and *Sugarcane yellow leaf virus* (SCYLV). The latter, SCYLV, is the most recently detected virus and is nowadays the only virus associated with Hawaiian sugarcane industry. It is the causal agent of yellow leaf syndrome (YLS) (now named Yellow leaf, YL) which was first reported from plantations on two Hawaiian Islands (Schenck, 1990). Few years later similar symptoms were observed in several other countries (Comstock et al. 1994) and dramatic yield losses were reported in Brazil (Vega et al. 1997). The symptoms are characterized by yellowing of leaf midribs followed by yellowing of the entire leaf blade (Fig.1a) and internode shortening of the green leaf top. The midrib yellowing may be intense or in some varieties may have a reddish tinge and is associated with sucrose accumulation in the midribs. The symptoms are best expressed when the crop is subjected to stress. Nevertheless, the pathogen can be present without the expression of symptoms. The virus particles were observed in the cytoplasm of phloem companion cells of sugarcane. The detection of SCYLV by Tissue–blot immunoassays (TBIA) also revealed that the sugarcane virus was associated with phloem (Fig.1c). The viral pathogen was classified as a luteovirus and was termed sugarcane yellow leaf virus (SCYLV) (Scaglisi and Lockhart, 2000). Todays analysis revealed that SCYLV belongs to *polerovirus* which is a member of the *luteoviridae* family and has a apparently arisen through

recombination between a *Polerovirus*, a *Luteovirus* and an *Enamovirus* (Moonan et al. 2000) (see Fig. 2). It is a +ssRNA-virus whose sequence contains 6 open reading frames.

SCYLV is spread from plant to plant by the common aphids *Melanaphis sacchari* and *Rhopalosiphum maidis*, whereas, the mechanical transmission have not been successful (Scagliusi and Lockhart, 2000). Within plantations the most important proliferation of SCYLV occurs by planting of infected internode pieces, the common practice in sugarcane industry.

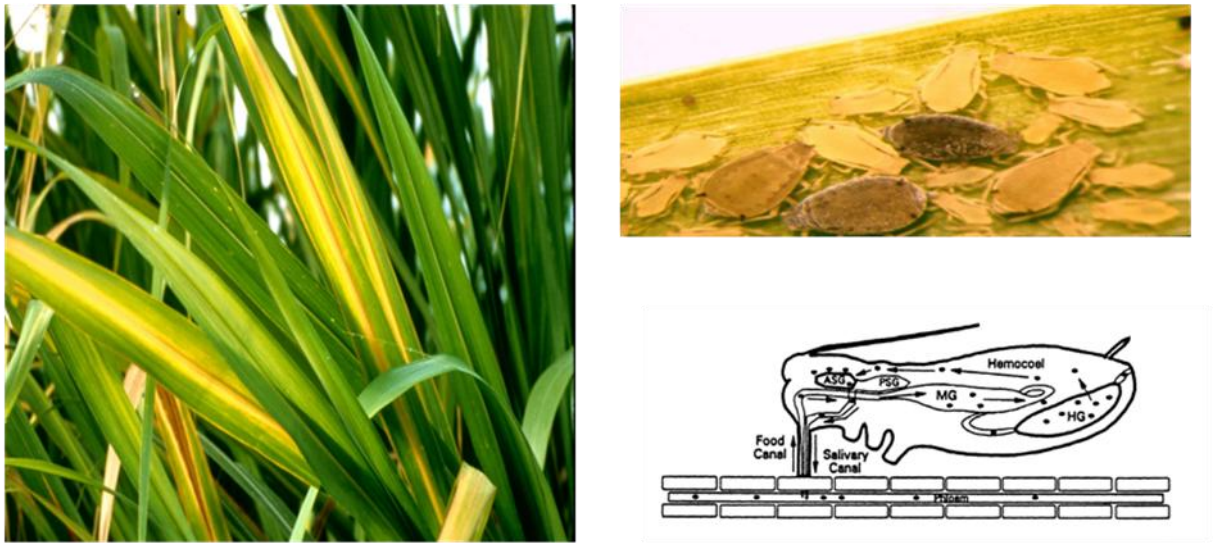

Fig.1 a) The symptoms of SCYLV, **b**) Transmission of SCYLV by aphids, *Melanaphis sacchari* (the graph are taken person.from Prof. Dr.Ewald Komor)

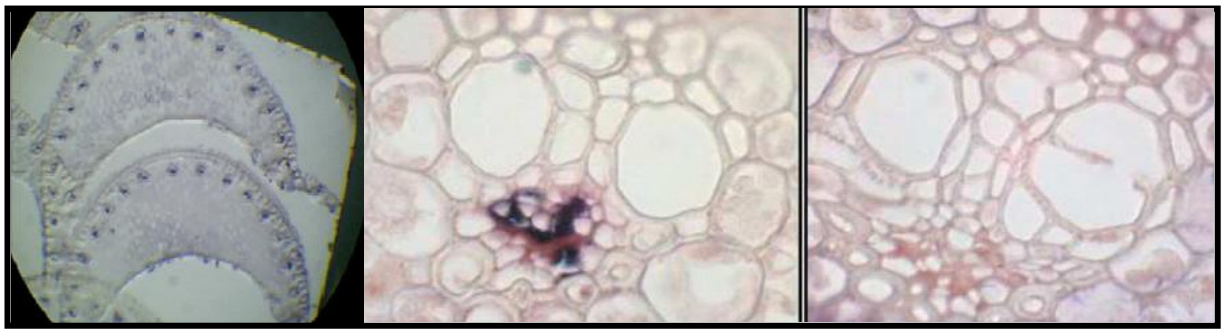

Fig. 2 Tissue–blot immunoassays (left) and in-situ RT-PCR of leaf midribs showing SCYLV within the phloem cells of an infected plant (middle) and as a control, of a not infected plant (the graph are taken from Lehrer et al., 2007)

The mechanical transmission of SCYLV has not been successful (Scagliusi and Lockhart, 2000). SCYLV can be eliminated by apical meristem culture (Fitch et al. 2001).The diagnosis of SCYLV depended on serological and molecular techniques such as tissue-blot immunoassay (TBIA), double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), and reverse transcription-polymerase chain reaction (RT-PCR) (Comstock et al. 1998;Schenck et al.

1997). The immunological hybridization techniques are not sensitive enough to detect proteins that are expressed at a low level, however they are very convenient for screening of large populations in the field. By applying the reverse transcription-polymerase chain reaction technique low levels of RNA can be detected. Nowadays an appropriate choice of parent varieties and discarding progeny with conspicuous YLS-symptoms during selection attempts to gain more resistant commercial cultivars.

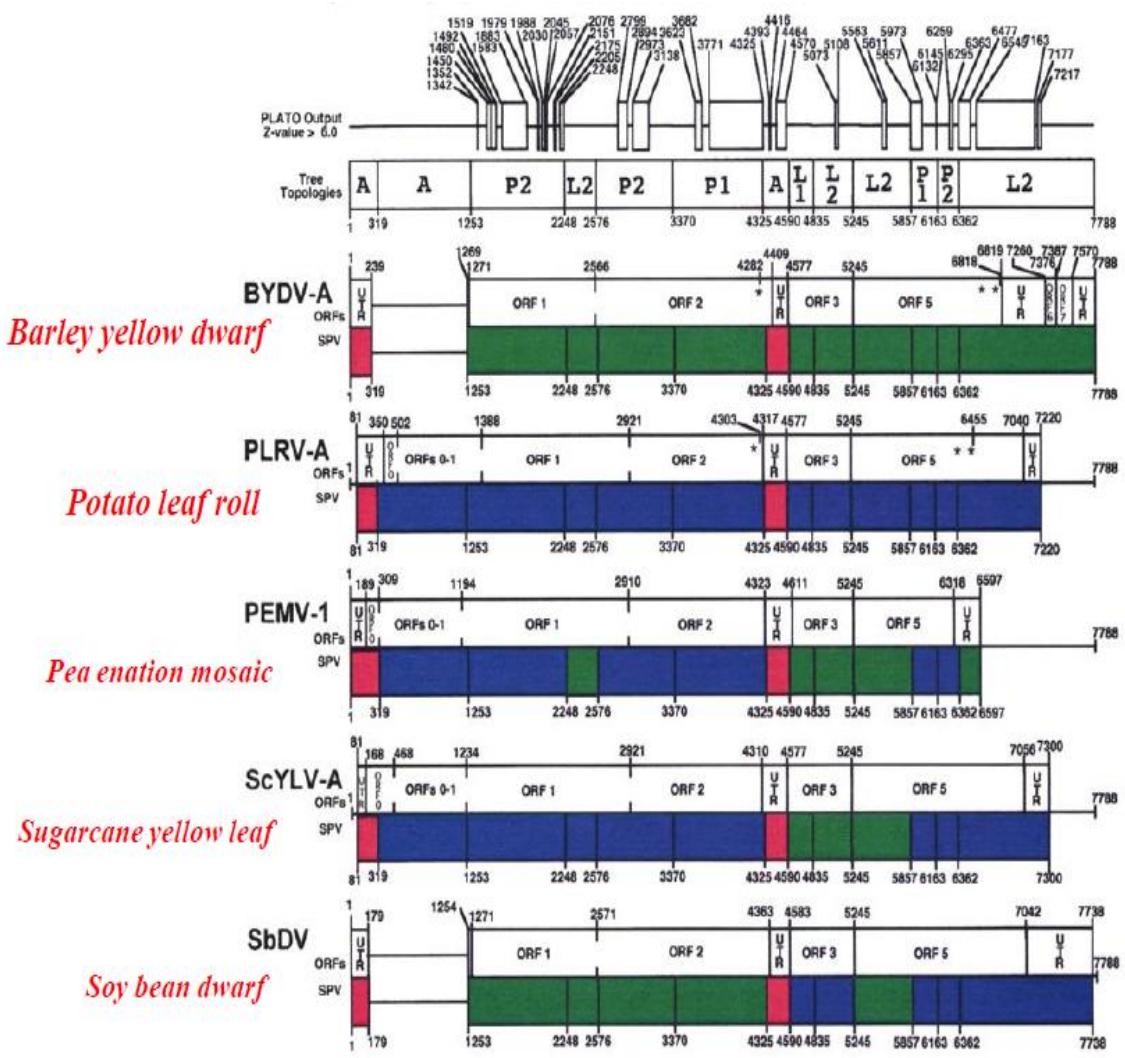

Fig. 3 A spatial Phylogenetic Variation (SPV) in the Luteoviridae family. The data and the graph are taken from Moonan et al., 2000.

Viral infection often affects carbon assimilation and metabolism in host plants. It is well known that the onset of leaf symptoms caused by plant viruses in their hosts depends on localised changes in the chloroplast structure and function. Direct evidence that virus infection affects the photosynthetic function over a broad spectrum has been obtained from studies with tobacco (*Nicotiana tabacum* L.) plants infected with Tobacco mosaic virus (TMV) genus Tobamovirus

(van Kooten et al. 1990 and Seo et al. 2000). Various results indicate that an increase in non-photochemical quenching of fluorescence and reduction in the fraction of open reaction centres leads to an increased reduction state of primary electron transport acceptor quinone A (QA). This suggests pronounced photoinhibitory processes following viral infection and symptom development. Photosynthesis reduction and chlorophyll degradation are however only the late stages of symptoms and may be caused by previous viral effects on plant cell metabolism. Studies with transgenic tobacco plants expressing the movement protein of TMV have shown effects of movement protein (without virus) on carbon metabolism, altering carbohydrate partitioning and plasmodesmal function between mesophyll cells (Balachandran et al. 1995; Lucas et al. 1996 and Olesinski et al. 1996). The source leaves of transgenic plants expressing the movement protein of Potato leafroll virus (PLRV), family *Luteoviridae*, genus *Potyvirus* showed accumulation of carbohydrates leading to a decrease in photosynthetic capacity, probably due to decreased expression of photosynthetic proteins (Herbers et al. 1997). These effects were strong in plants expressing the luteoviral movement protein in plasmodesmata of the phloem tissues, while in plasmodesmata of the mesophyll the effects were indistinguishable from the wild-type. The changes in carbohydrate status and viral resistance followed a protein level-dependent mechanism, whereas the plasmodesmal targeting and capacity of movement protein was not influenced by protein amount (Hofius et al. 2001). Corroborating these findings, Herbers et al. (2000) proposed a role for cell wall invertase in up-regulating the accumulation of soluble sugars and down-regulating photosynthesis, thus strengthening defence responses against viral attack.

b. *Sucrose transport in plants*

Although sucrose is commonly found in higher plant storage organs, it is generally at a low concentration, and starch is the predominant storage carbohydrate (Komor, 2000). Additionally, sucrose is the main transport molecule in most plants. Physicochemical properties of sucrose may play a role as transport sugar, because the viscosity of sucrose is relatively low at high concentrations such as in phloem sap, allowing high translocation rates (0.5 to 3 $m \times h^{-1}$). Furthermore, the disaccharide sucrose has a high chemical and biochemical stability due to its acetal-bond which covers the reducing ends of the two monosaccharide. Sucrose creates a high osmotic potential per carbon atom in the phloem sap, a key parameter for the mass transport efficiency within long tubes (van Bel, 1996).

The transport of sucrose from source organs to sink organs may follow a symplasmic pathway, moving from cell to cell via plasmodesmata. Alternatively, sucrose may move apoplasmically through the cell walls and intercellular spaces of the tissue. In most plants, the pathway from

source to sink is thought to involve a combination of both symplasmic and apoplasmic transport steps, depending on the tissue type and stage of development (Patrick, 1997 and Lalonde et al. 2003). Sucrose is produced in photosynthesizing cells, passes through the plasma membrane of these cells into the non-membrane bound area surrounding the mesophyll cells (apoplast) and is then actively transported into the sieve element system of the phloem. Estimates of the sucrose concentration in phloem of photosynthesizing leaves vary in the range of 0.3-0.8 M. While the total sucrose concentration in the producing cells is often as low as a few millimolar. This concentration step is consistent with some form of facilitated passage from the apoplast through a semipermable membrane and obviously requires the expenditure of metabolic energy (Komor, 2000).

Sucrose is the major mobile carbohydrate in the majority of higher plants. Our knowledge of sucrose translocation has increased considerably by the biochemical and molecular characterization of sucrose transporter (SUT) family in the last decade. Plant sucrose transporters (SUTs) belong to the glycoside-pentoside-hexuronide (GPH) cation symporter family (TC2.A.2) that is part of the *major facilitator superfamily* (MFS) (Chang et al. 2004). Transporters in the GPH family have the basic characteristics of MFS proteins: 12 transmembrane domains with N- and C-terminus in the cytoplasm. The first six transmembrane domains display some sequence similarities with the last six, supporting the idea that these transporters arose from at least one ancient gene duplication (Saier, 2000). Corroborating these findings, Henderson (1990) and Kaback (1992) described the hydrophobicity analysis of structure of an integral membrane protein with 12 putative transmembrane domains, with a central hydrophilic loop. Meanwhile, the GPH family contains members from bacteria, archaea and eukaryotes. Such as, melibiose permease from *E. coli* (Naderi and Saier, 1996), the α-glucoside transporter SUT1p from *Schizosaccharomyces pombe* (Reinders and Ward, 2001) and plantSUTs such as SUC2 from Arabidopsis (Sauer and Stolz, 1994; Chandran et al. 2003). Transporters within the GPH family that have been characterized so far transport glycosides by symport with a cation (H^+ or Na^+). Plant sucrose transporters were mainly associated with phloem loading. From sugarcane only one sucrose transporter (ShSUT1) was described. It is expressed in both leaves and stems, but most highly in the stem tissue accumulating sucrose (Casu et al. 2003). The protein was mostly localized at the layer of cells surrounding the bundle sheath but was absent from the phloem itself. Based on these findings, the ShSUT1 may play a role in retrieval of sucrose leaking from the storage parenchyma cells in the stem or alternatively in sucrose export into the storage parenchyma rather than in phloem loading (Rae et al. 2005a).

c. *Sucrose transport in sugarcane*

In sugarcane, the conducting cells of the leaf phloem are not connected to other cells of the leaf by plasmodesmata (Robinson-Beers and Evert, 1991). This suggests that phloem loading occurs from the apoplast in sugarcane. In phloem, sucrose moves out of the leaf and towards sink tissues. The movement of sucrose through transport phloem is thought to be driven by concentration gradients (VanBel, 2003). Sucrose transporters continue to be expressed in transport phloem and may act in retrieval of sucrose lost to the apoplast by leakage (Lalonde et al. 2003).

The role of transporters in the influx of sucrose during phloem loading has been well documented in contrast to their role in unloading and post-phloem pathways (Rae et al. 2005a). The gradient of sucrose concentrations suggests that post-phloem efflux from the symplast could occur by facilitated diffusion, movement through transmembrane pores, which has specificity for sucrose but which is driven solely by gradient of the substrate and not energized by direct or indirect consumption. The expression of sucrose transporters in the petiole tissues suggests that unloading involves an apoplastic step (Salmon et al. 1995). ShSUT1 was identified as sucrose transporter in stem of sugarcane, which is localized to tissues surrounding the stem vascular bundles (Rae et al. 2005b). Additionally, the sucrose transporter play a role in a tissue that predominantly supports symplasmic transfer is most likely to be in the retrieval of sucrose lost from the symplasmic continuum. This is analogous to the situation in the sieve elements of transport phloem in leaves, in which sucrose transporters continue to be expressed even though the sieve elements are connected by pores through the cell plates. The ShSUT1 sucrose transporter may be an important component of the retrieval mechanism. The expression of ShSUT1 in the cell layers at the boundary between these compartments may represent an additional biochemical barrier to apoplasmic sucrose movement through these layers (Rae et al. 2005a). It is also possible that ShSUT1 is involved in efflux of sucrose from the symplasm to the apoplasm at the boundary layer. It has been suggested that sucrose/H+ symporters may mediate sucrose efflux by facilitated diffusion in some circumstances (Lalonde et al. 2003).

The structure of sugarcane stem plays a role in the movement of sucrose from phloem to the storage parenchyma tissue. The vascular bundles of sugarcane stem are surrounded by a layer of fiber cells that become progressively lignified with development (Rae et al. 2005a). It has been suggested that, these layers can prevent and /or impede apoplastic movement of solutes during the period of sucrose accumulation. In agreement with these suggestions, it was found that these layers effectively form a barrier to apoplastic movement of water-soluble dyes during the period of sucrose accumulation and internode ripening (Jacobsen et al. 1992). Thus sucrose probably cannot reach the parenchyma cells from the phloem by apoplastic route. The presence of

plasmodesmatal connections suggests that the storage parenchyma cells obtain sucrose from the vascular bundle through symplastic passgae (Walsh et al. 1996). The pathway of sucrose into the storage parenchyma in the sugarcane stem is depicted in Fig. 3.

Plasmodesmata play an important role in long distance transport. Most plant cells (but not all!) are connected by plasmodesmata that allow small solutes and, under some conditions, macro-molecules to move between cells. Plasmodesmata serve an especially important role in the phloem. During the development of phloem, sieve elements (SE) and companion cells (CC) are formed from a common parent cell and they remain tightly connected by plasmodesmata. The plasmodesmata between sieve elements widen and form sieve pores in the sieve plates, thus creating a living tube through which the phloem sap can move rapidly. The companion cells retain the nucleus, vacuole and numerous mitochondria. There is evidence that specific messenger RNAs and proteins are produced in CC and are delivered to SE through plasmodesmata. It had been claimed that SUT1, sucrose/H^+ cotransporer, is localized in the plasma membrane of SE of Solanaceous plants, but the mRNA is made in CC (Kühn, et al. 1997). Thus SUT1 mRNA or protein and possibly other transporters has to traffic between the two cells by receptor-mediated transport through plasmodesmata.

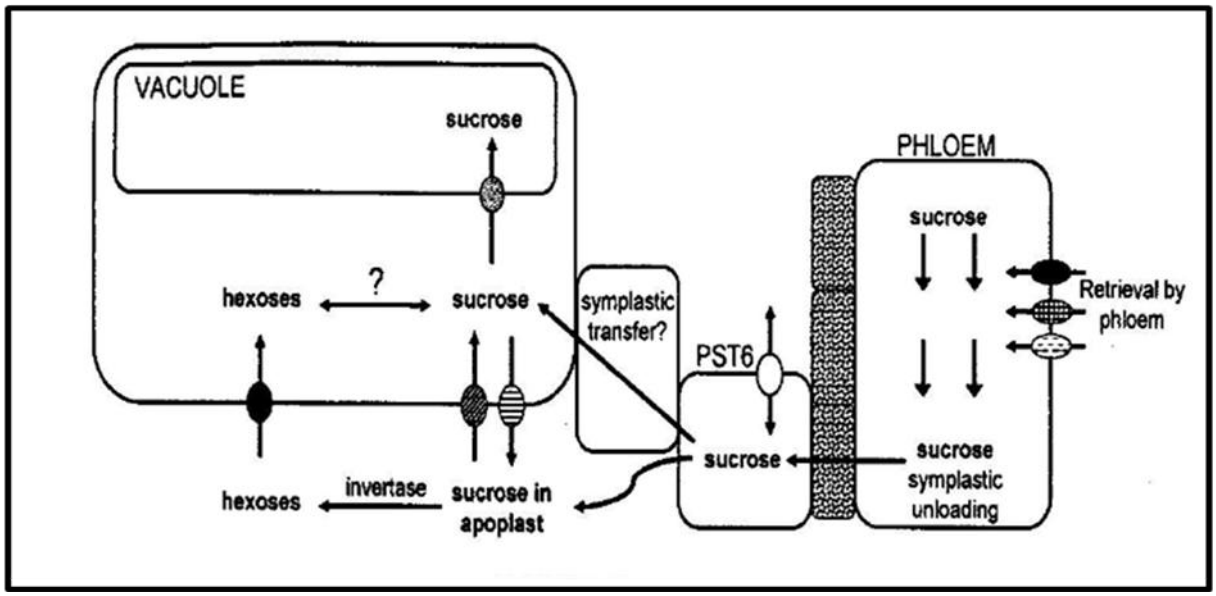

Fig. 3 Possible routes of sucrose into the storage parenchyma of the sugarcane stem. One way is symplastic unloading through plasmodesmata by cell-to-cell connections without any apoplastic step. Another possibility is the unloading of sucrose into the apoplast, followed by hydrolysis by acid invertase and subsequent uptake of the resulting hexoses into the sink cells. This step would then be followed by resynthesis of sucrose in the cells. A third possibility is the unloading of sucrose into the apoplast followed by uptake of intact sucrose into the cells. The data and the graph are taken from Rae et al. (2005b).

Besides the pathway of sucrose from stem phloem to stem storage parenchyma, there is also a metabolic cycling of sucrose. Sucrose is synthesized by two alternative ways. Sucrose-phosphate

synthase synthesizes sucrose-phosphate from UDP-glucose and fructose-phosphate.The following phosphatase step leading to sucrose shiftes this reaction sequence strongly towards sucrose synthesis. The other way, sucrose synthesis from UDP-glucose and fructose by sucrose synthase is relatively reversible and may be a means to provide sufficient levels of UDP-glucose from sucrose for cell wall synthesis (e.g. for callose synthesis in sieve tubes, which are devoid of invertase). SPS and SS are present in sugarcane storage parenchyma (Zhu et al. 1997). Invertase is the major enzyme responsible for sucrose hydrolysis. There are several isozymes present in storage parenchyma, a cell wall-bound acid invertase, a cytosolic neutral invertase and a vacuolar acid invertase. The balance between these enzyme activities changes during internode maturation and is thought to be an important factor in determining sucrose yield of sugarcane varieties (Zhu et al. 1997 and Lingle,1989). Sugarcane industry is interested in increased concentration of sucrose as the key objective for sugarcane improvement programmes.

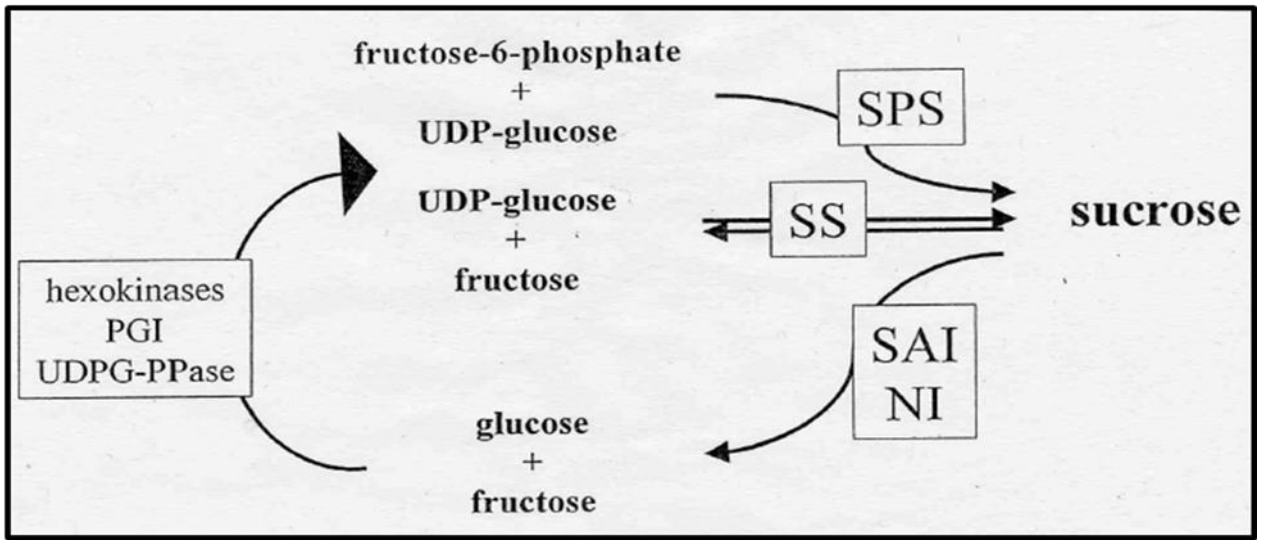

Fig. 4. The cycle of sucrose in sugarcane, enzymes and metabolites: SPS; sucrose phosphate synthase, SS; sucrose synthase, SAI; soluble acid invertase, NI; neutral invertase, PGI; phosphoglucomutase, UDPG-PPase; UDPglucose pyrophosphorylase. All these enzymes are supposed to be cytosolic with exception of the soluble acid invertase, which is vacuolar. In addition a cell wall bound acid invertase will hydrolyze apoplastic sucrose. The data and the graph are taken from Komor (2000).

Aims of the present study

Sugarcane is an economically important crop species for targeted breeding and an interesting model to study sucrose transport as well. The worldwide distribution of sugarcane yellow leaf virus in sugarcane plantations makes it interesting to study the genetic diversity of sugarcane yellow leaf virus. The best studied effects of SCYLV on the physiology of sugarcane were made on Hawaiian cultivars, however a molecular characterization on the virus in Hawaii was lacking. The objectives of this study were to determine and characterize SCYLV in Hawaiian varieties,

resistant and susceptible ones, to investigate possible sequence divergences and the genetic relationships between SCYLVs from susceptible and resistant Hawaiian cultivars. (In addition a few cultivars from Middle-East, the home country of the author, were tested for SCYLV).
It had been suggested that the viral effects which ultimately lead to symptoms may be connected to reduction of sucrose export from the leaves. ShSUT1, which is expressed in leaves and stems may play an important role in the accumulation of sucrose in maturing stem. It should be tested, whether it is affected by SCYLV-infection. ShSUT1 transcripts level in different tissues (shoots of seedling stage, source leaves and storage tissues) of SCYLV-infected and not-infected sugarcane plants were determined. In addition carbohydrate profiles were determined to evaluate the physiological status of the infected plant.

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3. Synopsis

This thesis comprises five publications which are presented in chapters 4 to 8.

3.1. Selection of susceptible and resistant cultivars for SCYLV

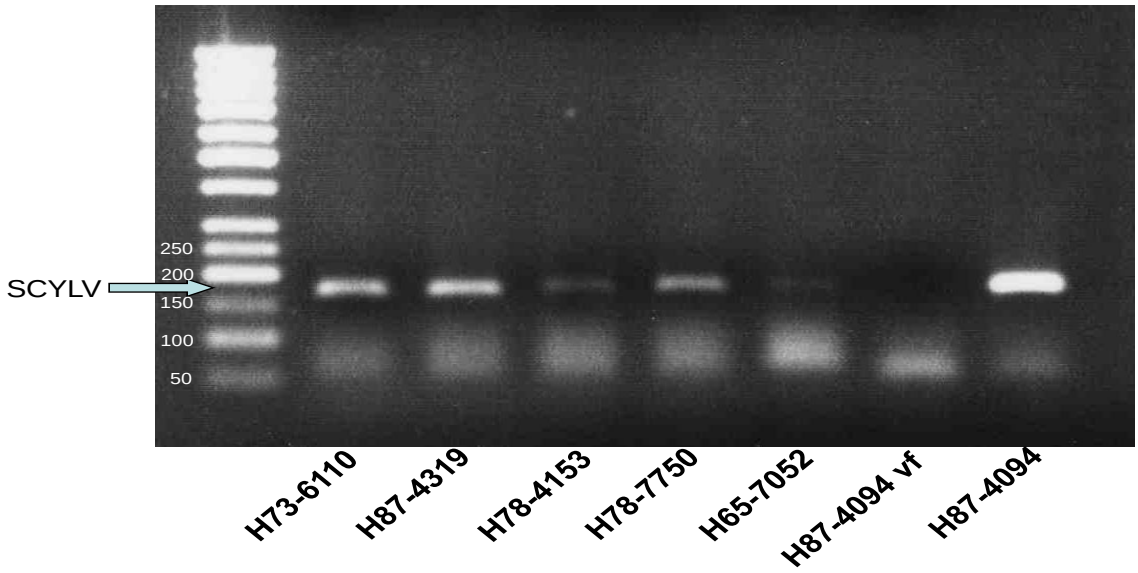
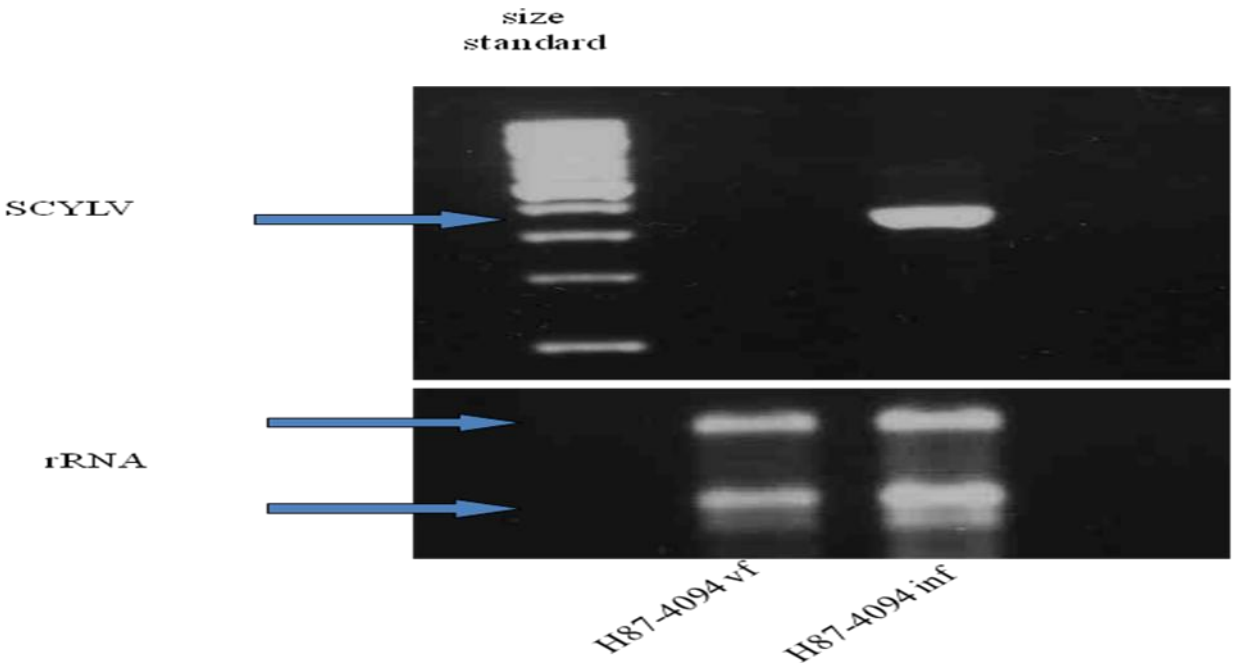


Fig. 1 RT-PCR of RNA-derived cDNA from source leaves of different cultivars of sugarcane. RNA from leaves of five representative cultivars was extracted, transcribed to cDNA and amplified by RT-PCR. H73-6110 and H87-4094 are susceptible, H65-7052 is moderately susceptible, and H78-4153, H87-4319 and H78-7750 are resistant cultivars. The virus-free clone of H87-4094 was used as a negative control. The amplified SCYLV was 165 bp long , the size standard was a 50-bp DNA ladder (left).

A previous survey using Tissue–blot immunoassays (TBIA) had identified SCYLV-susceptible and SCYLV-resistant cultivars. Cultivars that expressed fluctuating levels of virus titer were called moderately susceptible. Tests for SCYLV by RT-PCR partly confirmed the different titers of virus in the different cultivars; however, the so-called resistant cultivars (H78-4153, H87-4319, H78-7750) that had appeared without SCYLV in TBIA, had SCYLV (Fig. 1), though at a much lower titer than, for example, H87-4094. H65-7052 appeared nearly virus-free, possibly the leaf had been sampled in a virus-poor phase. Nine cultivars were selected for a test of carbohydrate status of sugarcane plants at harvest time (16 months), the same cultivars that had been used previously in an extended yield test in different Hawaiian fields.

3.2. Maintenance of SCYLV-infection in sugarcane stalks (seedling stage)

Fig. 2 *RT-PCR for SCYLV in virus-free and infected cv. H87-4094 after 12-16 cycles of replanting, with rRNA as loading control.* Virus-free and infected plants of H87-4094 were grown in the greenhouse outside of insect-tight cages. RNA from leaf samples was extracted and amplified with SCYLV-specific primers by RT-PCR. The reaction products were separated on gels and stained with ethidium bromide. Length of amplified SCYLV was 165 bp, the size standard was a 50 bp DNA-ruler from Fermentas (St. Leon Rot, Germany).



Sugarcane is propagated vegetatively by cuttings. It was important to show whether SCYLV is propagated by seed pieces to successive generations. Cultivars which were imported as one-node seed pieces from Hawaii in 2001, were grown in the greenhouse at the Bayreuth University. The plants were cut 1-2 times per year and each time regenerated from seed pieces. The cultivars, which were SCYLV-infected 8 years ago when collected in the field, still contained SCYLV after the 12-16 cycles of replanting in the greenhouse (Fig. 2). When seed pieces of infected plants were germinated in an insect-tight cage, the freshly emerged leaves already contained SCYLV (Fig. 3). Virus-free plants of the susceptible cultivar H87-4094 remained virus-free in the Bayreuth greenhouse even when outside of insect-tight cages over several years (Fig. 2), which indicates that the greenhouse is free of SCYLV-vectors. The presence of SCYLV in the infected cultivars over so many replanting did therefore not originate from *de novo* infection in the greenhouse.

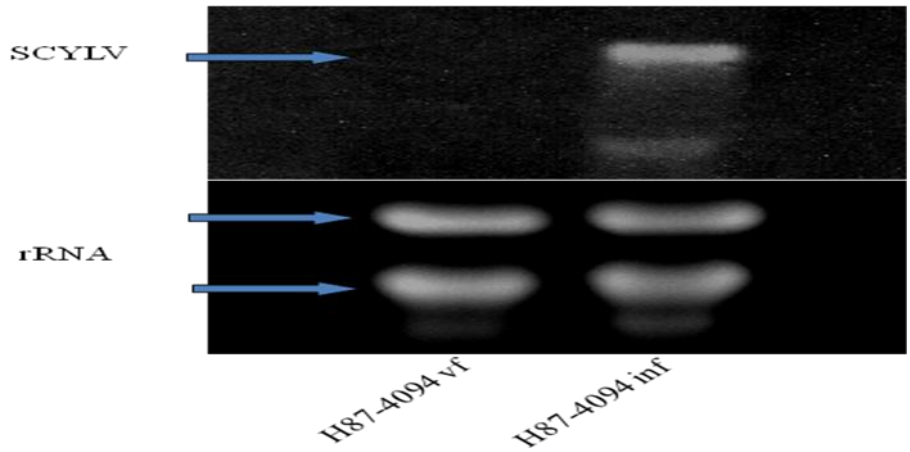


Fig. 3 *Northern Blot of RNA from freshly germinated seed pieces.* Seed pieces of cv. H87-4094 were germinated for 3 weeks in insect-tight cages and RNA was extracted from the freshly emerged leaves. H87-4094 inf = infected cv. H87-4094, H87-4094 vf = virus-free plants of H87-4094. The RNA of SCYLV and, as a loading control, of rRNA is indicated by arrows.

3.3. Molecular characterization of Hawaiian *Sugarcane yellow leaf virus* genotypes and their genetic diversity

Phylogenetic relationship of the Hawaiian SCYLV-isolates.

Twenty-five Hawaiian amplicons were used for phylogenetic analysis together with sequences from the GenBank data base (http://www.ncbi.nlm.nih.gov/Genbank/index.html) (Table 2, see chapter 6). Since the SCYLV-genome is a recombination product of two *Luteoviridae* viruses, the phylogenetic relationship of fragments A-D and YLS were separately constructed to visualize possible sequence segments where the Hawaiian strains may have diverged from other so far sequenced strains.

Nucleotide sequence corresponding to fragment A (comprising partial ORFs 0 and 1). Forty-three virus isolates were used in phylogenetic analysis (four isolates from current study; Haw73-6110a, Haw73-6110b, Haw87-4319 and Haw87-4094). These sequences were found to be distributed into three major groups by (Fig. 4b, see chapter 6). Cluster A1 contained 19 SCYLV sequence isolates from different origins, such as USA, Brazil, Taiwan, Cuba, China, Australia, India, Malaysia and south Africa exception one isolate from Réunion (REU42). However, the Hawaiian virus isolates and Peru group were distributed in cluster A2. It may be important to mention that two sequences obtained from different RNA extractions of isolate Haw73-6110 were identical. Despite of the Hawaiian isolates were clustered in one group, but our Hawaiian

virus isolates (Haw73-6110a and b, Haw87-4319 and Haw87-4094) were divided into different subgroups. Haw87-4094 was relatively close to SCYLV-F (cluster A1) and isolate Haw87-4319 showed 91.2% identity with other Hawaiian isolates. Hawaiian SCYLV isolates are closely related to Peru and Brazil groups. The sequence identity among Hawaiian isolates ranges between (91.2% and 98.9%). The majority of Réunion virus isolates used in phylogenetic study and obtained from GenBank were clustered into unique group (A3), in which REU-YL1a and REU-YL1b were identical.

With regards to fragment B (partial ORF2), thirty-seven sequences isolates of SCYLV in which four were amplified in this study were classified into three clusters by phylogenetic analysis (Fig. 4c). Hawaiian virus isolates (Haw73-6110, Haw87-4319 and Haw87-4094) were distributed within cluster B1 under a unique subgroup. Furthermore, the sequence identity between Haw87-4319 and other Hawaiian isolates were between 89.5% and 90.3%. As expected, the Réunion isolates were grouped all together in cluster B2. Cluster B3 contained only two Cuban virus isolates (CUB-YL1& CP52-43) with 98.7% identity.

Six fragments YL0, A, B, C, D and YL5 from different genome locations of SCYLV were amplified for three virus isolates (Haw73-6110, Haw87-4319 and Haw87-4094). These six fragments covered the six ORFs of SCYLV genome. Phylogenetic analysis (Fig. 4) was performed for three Hawaiian isolates complete genome, the other isolates PER, REU, SCYLV-A, SCYLV-F, SCYLV-IND, China and Brazil were obtained from GeneBank database. Thirteen complete sequences of SCYLV genome were analyzed by phylogenetic analysis and distributed into three groups. Group 1 (HAW/PER) included two subgroups, which contains Hawaiian and PER isolates with bootstrap value 89%. The identities within Hawaiian isolates vary between 97%-99%. Strain SCYLV-F was relatively close to Haw73-6110. Group 2 (BRA) formed by various origins isolates (Brazil, China, India and USA). Strains SCYLV-A and SCYLV-IND were 100% identical. REU group was clustered in group 3 with 100% identity. Thus the whole genome reflects the impression already obtained from the alignment of partial sequences which are available in much larger numbers

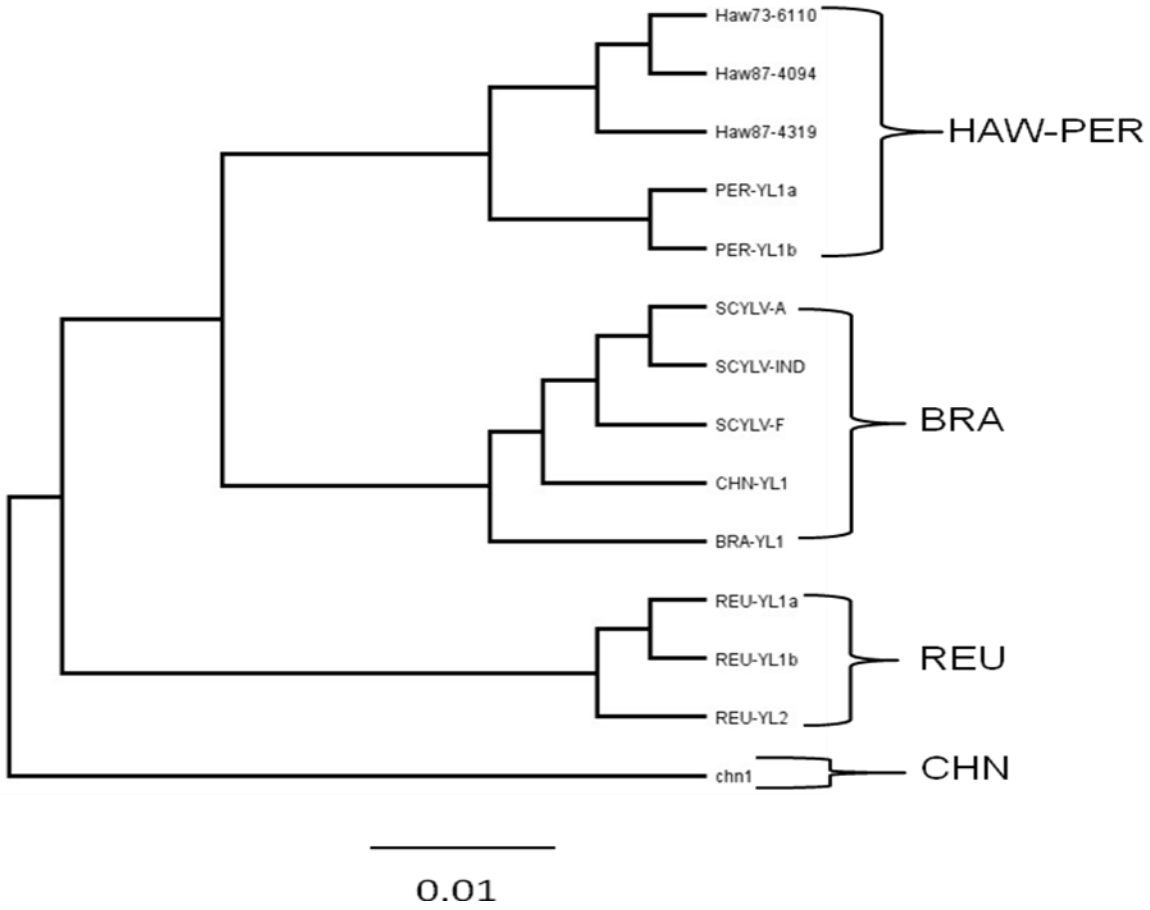


Fig. 4 The genetic diversity of complete nucleotide sequences ORFs 0-5 of sugarcane yellow leaf virus isolates from different geographical origin assessed with Geneious program, UPGMA phylogenetic tree.

Recombination analysis

To understand the taxonomic and evolutionary positions of isolates Haw73-6110, Haw87-4319 and Haw87-4094 within the family *Luteoviridae,* sequences of these three viruses and other SCYLV isolates were compared to well-characterized *Luteoviridae* members. The 26 sequences were analyzed and the results indicated the SCYLV isolates were clustered in a unique cluster and more related to members of the genus *Polerovirus* than to *Luteovirus* (Fig. 5). This result confirmed our assumption of the classification of SCYLV population as three groups. Sequence alignment between genus *Polerovirus*, *Enamovirus* and *Luteovirus* revealed a 100% sequence identity.

Taken together, the above results of phylogenetic analysis either in SCYLV isolates or included the *Luteoviridae* family indicate that the Hawaiian virus isolates and other SCYLV isolates should be considered as definitive members of the family *Luteoviridae* and genus *Polerovirus*. Also, the recombination events may play an important role in generating genome diversity.

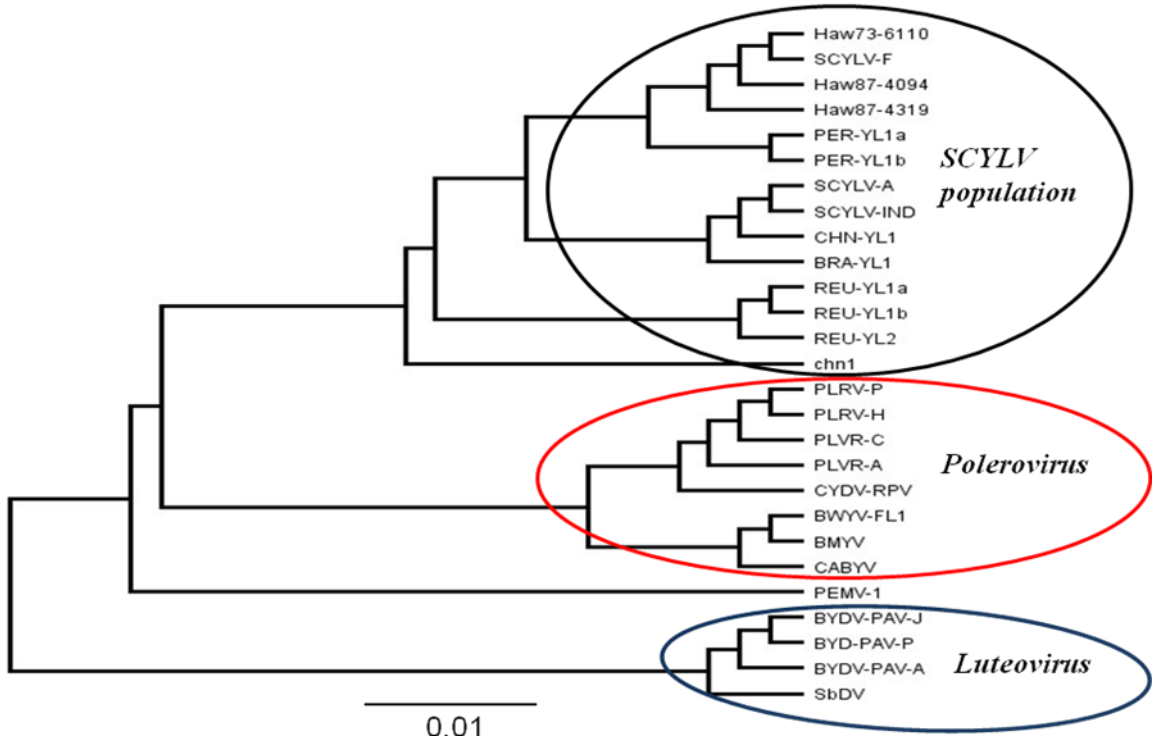


Fig. 5 Phylogenetic relationships among viruses from the family Luteoviridae. The nucleotide sequences were aligned with CLUSTAL W and the tree was constructed with Geneious program and UPGMA method.

Deletion/insertion in ORF1

The nucleotide sequences from 2 susceptible cultivars (H78-6110 and H87-4094) and from the resistant cultivar (H87-4319) showed a lack of 48 to 54 nucleotides in the susceptible cultivars (Fig. 6). A 51 nt deletion was detected in fragments A and B of cultivar H87-4094 corresponding to nucleotides 1686 to 1736 of SCYLV (NCBI accession NC_000874, Moonan et al. 2000). In contrast, a 48 nt deletion was detected in two independent A fragments obtained from cultivar H73-6110 corresponding to nucleotides 1686 to 1733 of SCYLV (see above). However, sequence analysis of fragment B amplified from total RNA of the same cultivar H73-6110 exhibited a 54 nt deletion corresponding to nucleotides 1681 to 1734 of SCYLV (see above). Since these deletions were detected in independent amplification products of these two sugarcane cultivars, they did most likely not result from amplification and cloning artifacts or from sequencing errors. In addition, the detection of a 48 nt and a 54 nt deletion in amplification products from cultivar H73-6110 might indicate the presence of at least two SCYLV genotypes in this plant line. RT-PCR with primers flanking this particular region yielded in amplification products of the expected size; 409 bp from the resistant cultivar and about 359 bp from the susceptible cultivars (Fig. 6). The deletion in SCYLV from susceptible cultivars lies in the ORF1

for a “multifunctional protein” which is thought to be involved in suppression of gene silencing, and at a cleavage point of RNA-dependent RNA polymerase (RdRp, ORF1 to ORF2).

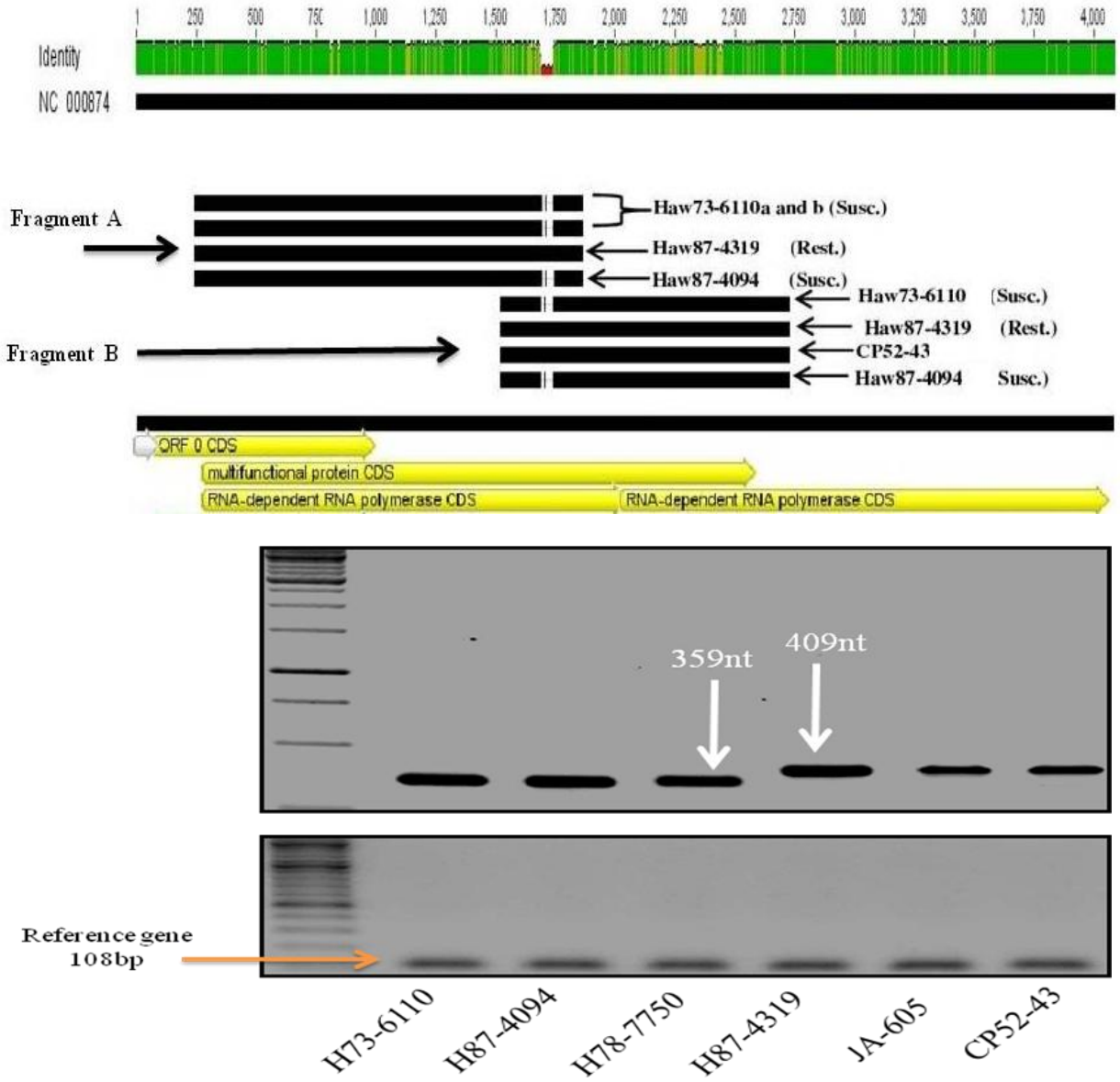


Fig. 6 Sequence gap in SCYLV from susceptible cultivars (top) and RT-PCR of the sequence segment containing the deletion (bottom). Top: Location of sequence gap in SCYLV from susceptible cultivars versus SCYLV from resistant cultivar and ORFs for coded proteins. The gap was in overlap of fragments A and B, the deletions were in susceptible cultivars only. Bottom: RT-PCR of the sequence segment containing the deletion. Primers YL1FOR and YL1REV were designed to amplify the sequence nucleotide. RNA-preparations from susceptible (H78-6110 and H87-4094) and resistant cultivars (H87-4319) were used as templates. Lower panel loading control (25srRNA) 108bp (M: DNA size marker).

The amino acid sequences of RNA-dependent RNA polymerase (RdRp) from fully sequenced SCYLV-strains showed lower sequence identities in the first half and high identity in the second half of the protein (Fig. 6, see chapter 6). The 16 aa gap (48 nt deletion) and 17 aa gap (51 nt

deletion) of the two isolates Haw73-6110 and Haw87-4094 lies just in between of these two halves (the 18 aa gap of the 54 nt deletion is not shown). The deduced amino acid sequences of the capsid protein (CP) obtained from all the isolates expressed almost identical amino acid sequences (97-100%, not shown).

3.4 Sequence deletion in *Sugarcane yellow leaf virus* genome and their effect on the diversity of virus population

Significance of the deletion/lacking in ORF1

In order to understand the effect of deletion sequence in the replication of SCYLV genome, two experiments have been designed. The first experiment was inculcated the sugarcane cultivars susceptible and resistant with aphids *Melanaphis sacchari*, which is the transmission vector of SCYLV. Interestingly, the results of RT-PCR revealed that, nine cultivars carried the sequence deletion out of twelve, while three cultivars have a complete sequence/no stretch lacking. In addition, the aphids carried also a deletion (Fig. 7a and b).

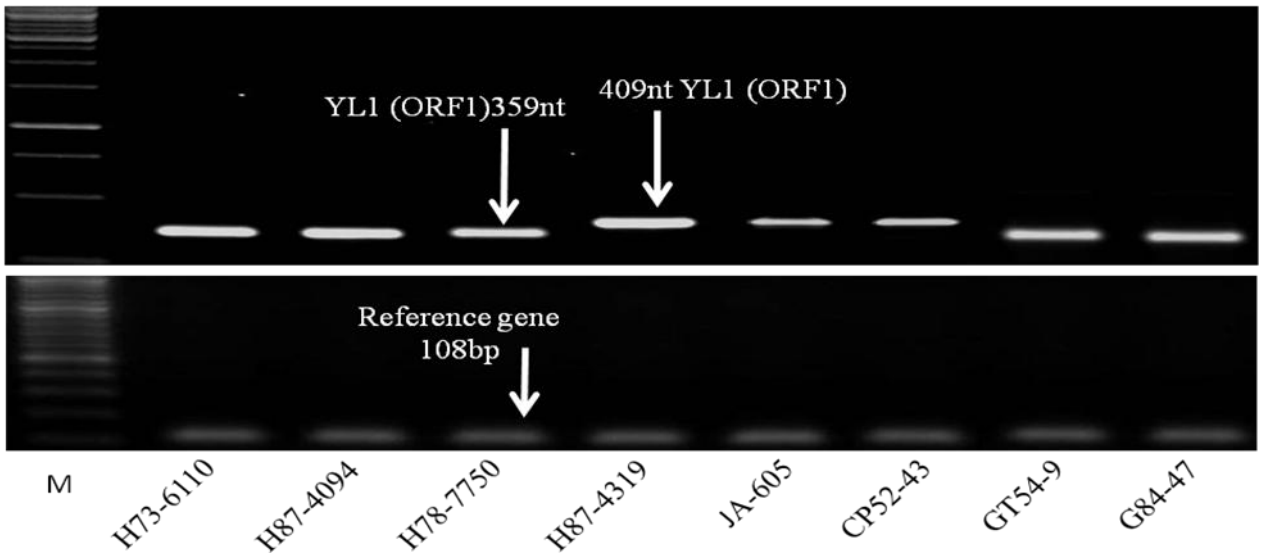


Fig. 7a RT-PCR of the sequence segment containing the deletion. Primers YL1FOR and YL1REV were designed to amplify the sequence nucleotide from 1211 to 1620 nucleotide

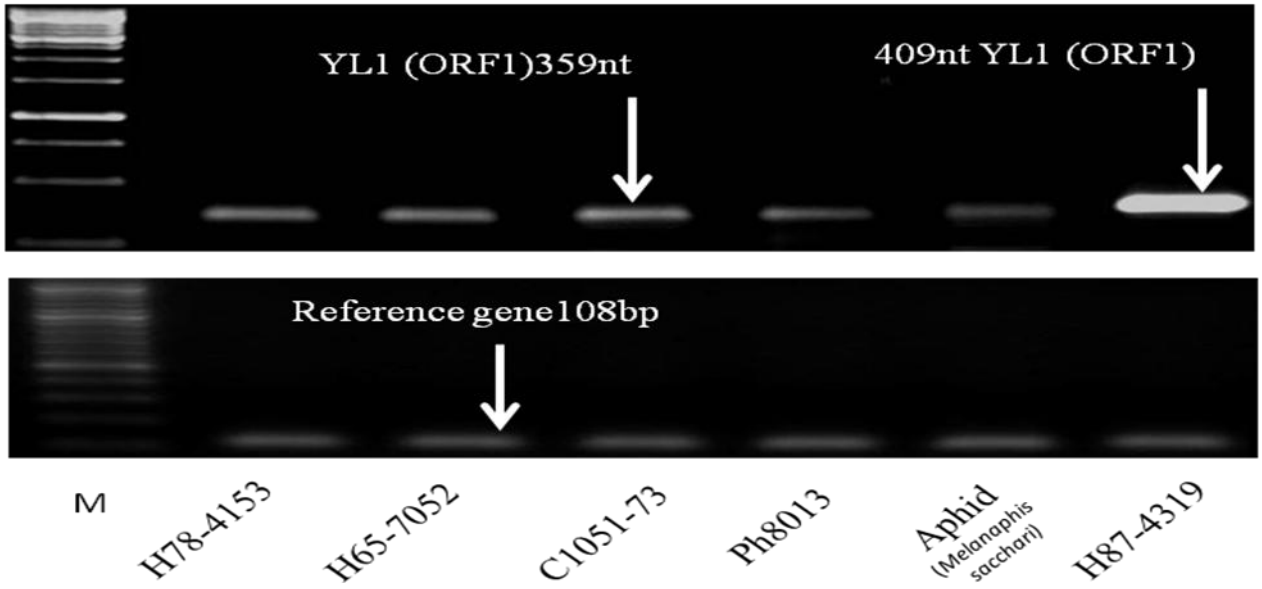


Fig.7b RT-PCR of ORF1 sequence containing the deletion. YL1 primer was designed to cover the deletion part sequence to investigate if the cultivars under study have a deletion or not.

Concerning to the second experiment, the cultivars H78-4153, H65-7052, Ph8013 and H87-4094_virus free were inoculated with viruliferous aphids *Melanaphis sacchari* in an insect-tight cage. The aphids have been fed on the cultivar have a complete sequence (no stretch lacking), H87-4319. The results of RT-PCR showed that, the cultivar H87-4094_virus free was infected by SCYLV strain contain a complete genome without deletion region, in addition the aphids also has the same expression. In contrast, the cultivars H78-4153, H65-7052 and Ph8013 were carried SCYLV with deletion about 50bp missing (Fig. 8). We mentioned that these cultivars has been infected by the deletion virus strain before inoculation with the resistant cultivars and /or complete genome. The results of the second experiment indicated that the aphids carried and transmitted the virus particles as it is. Reasonable that the virus particles are not able to replicate into the aphid organs, but there is other proposes that the virus could replicate in the plant.

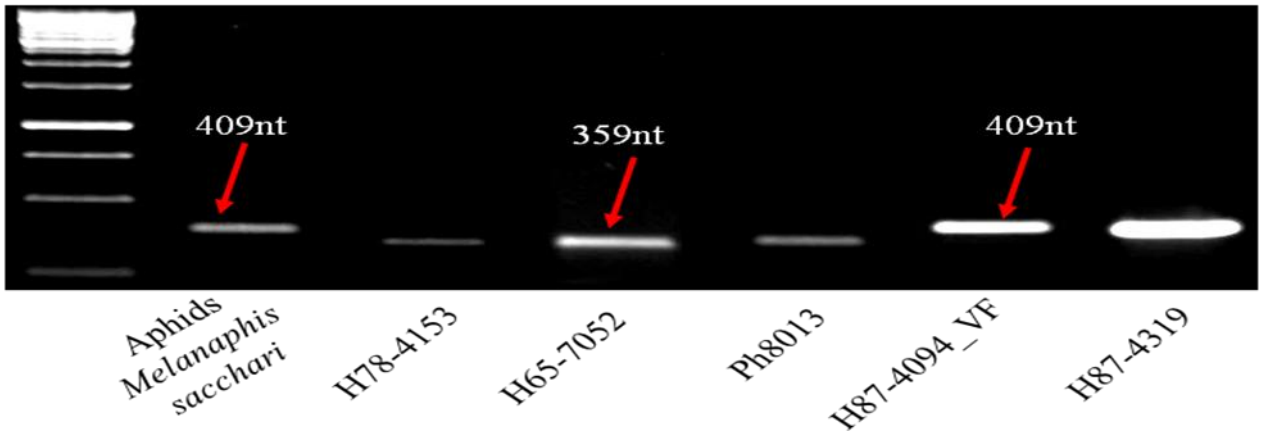


Fig. 8 detection of the inoculated sugarcane cultivars with the resistant cultivar, which have no sequence lacking by RT-PCR, using YL1 primers.

3.5. Expression of sucrose transporter (ShSUT1) in a Hawaiian sugarcane cultivar infected with Sugarcane yellow leaf virus (SCYLV)

Changes in the sugar composition

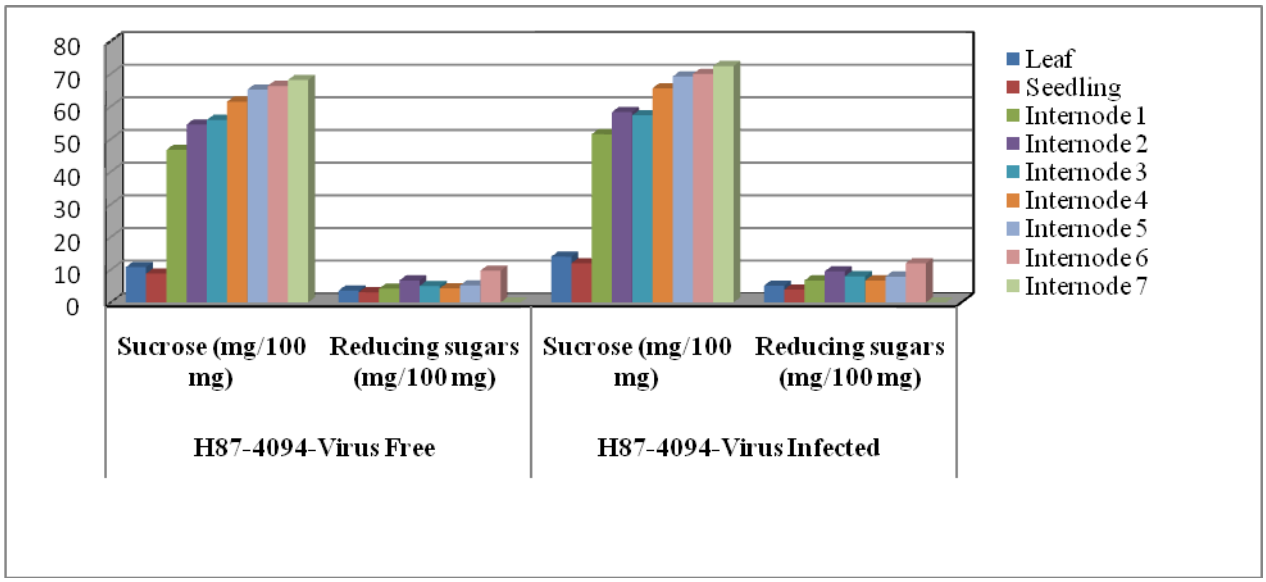


Fig. 9 Carbohydrate contents in leaves, shoots of seedling stage and internodes of healthy and sugarcane yellow leaf virus (SCYLV) infected sugarcane (H87-4094-virus free and H87-4094-virus infected) plants. The sugars were separated using HPTLC.

The sugar profile in leaves (source leaf), shoots (seedling) and internodes of healthy and sugarcane yellow leaf virus (SCYLV) infected plants were assessed for comparison with the pattern of expression of the sucrose transporter ShSUT1A (Fig. 9). In order to verify the impact

of SCYLV on the metabolism of carbohydrates in sugarcane tissues, the contents of sucrose and reducing sugars were determined using HPTLC. Sucrose contents in leaves were increased by SCYLV infection. Relative to the leaves of healthy plants, reducing sugars were the most accumulated sugars in the leaves of infected plants. The reduction in sucrose in the shoots tissues of seedling stage was found in the healthy plants, followed by reducing sugars. On the other hand, the accumulation of sucrose in storage tissues (internodes) was increased by SCYLV infection, compared with healthy plants. The high concentration of accumulated sucrose was found between internodes 5-7, whereas, no accumulation for reducing sugars was found in internodes 7.

Transcripts of ShSUT1 in different tissues of sugarcane

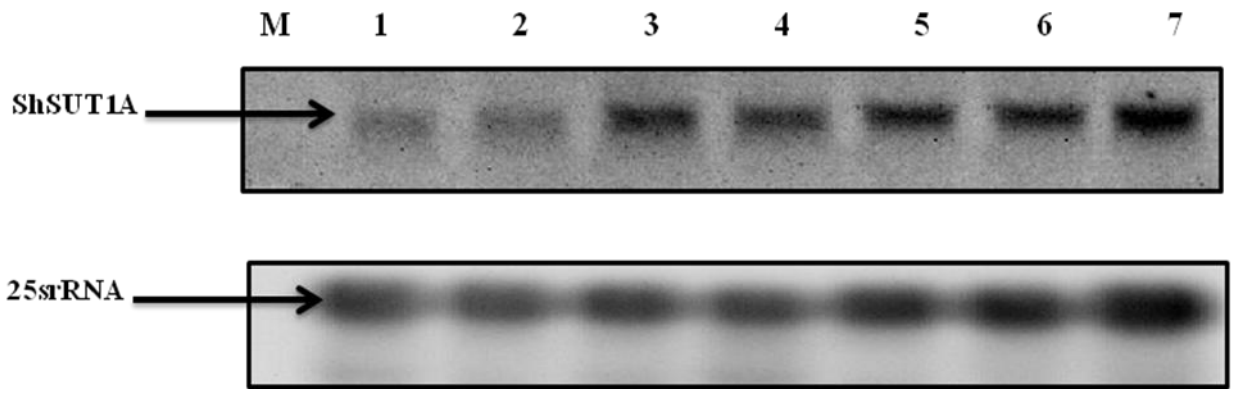


Fig. 10 Abundance of transcripts of *ShSUT1A* in sugarcane cv. H87-4094 virus free. RNA was extracted from different internode tissues (from 1 to 7) and was hybridized to a probe of the *ShSUT1A* cDNA. The lower panel shows the same membrane probed for ribosomal RNA to demonstrate RNA loading

The hybridization of RNA contained virus free sugarcane as seen in Fig. 10. It was noted from the result of RNA hybridization that the transcript expression was relatively higher in the maturing internodes (5-7) than in the younger internodes (immature).

Phylogenetic relationships among the sucrose transporters (SUTs)

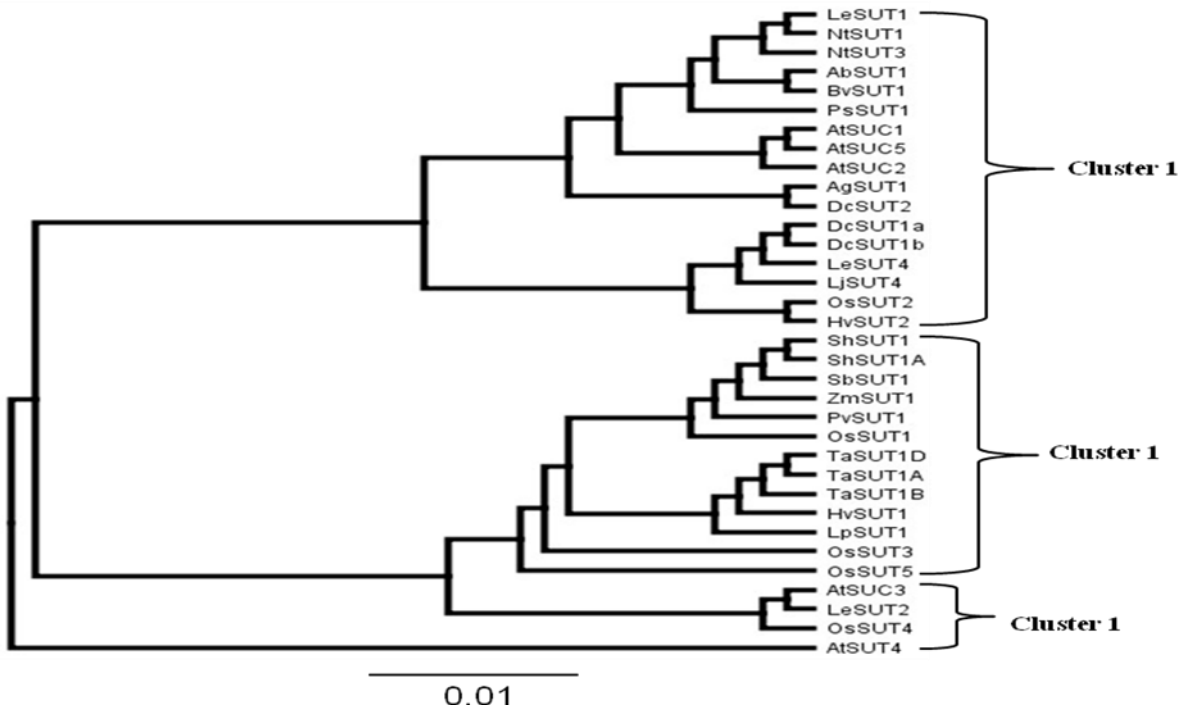


Fig. 11 Phylogenetic tree was constructed based on nucleotide sequence alignments of 34 plant sucrose transporter (SUTs). The tree was constructed with Geneious program and UPGMA method

Phylogenetic relationships among the sucrose transporters (SUTs) subfamilies were determined by thirty-four nucleotide sequences and the sequences were aligned with CLUSTAL W. Dendogram constructed from nucleotide sequences and distributed into three major groups by phylogenetic analysis (Fig. 11). Cluster 1 contained 17 SUTs sequences from different origins (*Oryza sativa*, *Apium graveolens*, *Asarina barclaiana*, *Beta vulgaris*, *Daucus carota*, *Arabidopsis thaliana*, *Lycopersicon esculentum*, *Nicotiana tabacum*, *Hordeum vulgare*, *Lotus japonicas* and *Pisum sativum)*. The similarities among sequences were varied 73 -100%. Moreover, the cluster 1 included dicot SUT4 (LeSUT4 and LjSUT4), bootstrap of 73% and dicot SUT1 (LeSUT1, DcSUT1, NtSUT1 and AtSUC). Monocot SUT1 were clustered into cluster 2 included 13 SUTs with high bootstrap from 83 to 100%. ShSUT1A was closed to ShSUT1 with 99.9% identity, additionally the OsSUT5 showed 99.5% identity with other monocot SUT1. Cluster 3 formed by four sucrose transporters of dicot SUT2 (AtSUC3, LeSUT2, OsSUT4 and AtSUT4), 78.3% bootstrap with the monocot SUT1. The identity between AtSUC3 and LeSUT2 was 98.8%.

3.6. Quantitative multiplexed gene expression

Quantitative gene expression analysis would give more accurate relative quantitative information on the ratios of virus titre and sucrose transporters (SUTs) in different cultivars, during different plant stages. We determined four different genes of SCYLV, two genes of sucrose transporters and one gene of sucrose phosphate synthase (SPSII) in one multiplex using GenomeLab GeXP Genetic Analysis System. The plants under study were (H73-6110, H87-4094-vinf, H87-4319 and H87-4094-vf). The materials were taken from different plant tissues; sink leaves, source leaves and mature internodes (8 to 9). The results of RT-qPCR by GeXP (Fig. 2, see chapter 8) revealed that the four different genes of SCYLV were highly expressed in the sink leaves of seedling stage, source leaves and mature internodes tissues (Fig. 2 see chapter 8). Furthermore, the ORF0/1 was more highly transcript compared with other genes in all infected cultivars at different plant stages. ORF0 is considered highly conserved region in SCYLV genome. In potato leaf roll virus ORF0 was found to be effective in symptom development. Hence in SCYLV it could be useful when ORF0 used as a diagnostic region. Additionally the ORF3/4 which related to capsid protein and movement protein was slightly low expressed in sink and source leaves, for unknown reasons. Whereas, the transcripts of ORF2 which encodes for RdRp were constant in all infected cultivars at sink leaves of seedling stage. But the expression of RdRp was variable in the source leaves and mature internodes.

The RT-qPCR using GeXP analysis showed that sucrose transporter (ShSUT1) was a higher transcript expression than the sucrose transporter (ShSUT4) in the sink leaves and source leaves with all tested cultivars (Fig. 4 see chapter 8). The highest levels of sucrose phosphate synthase (SPSII) transcript expression were present in the mature internodes in all tested cultivars. Furthermore, the SPSII was expressed in different plant tissues (photosynthetic and nonphotosynthetic tissues) (Fig. 4 see chapter 8)

3.7. Individual Contribution to Joint Publications

The results presented in this thesis were obtained in collaboration with others and published or submitted as indicated below. In the following the contributions of all the co-authors to the different publications are specified. The asterisk denotes the corresponding author.

Chapter 4

This work is published in ***Journal of General Plant Pathology*, 2010, 76: 62-68,** under the title, **"Carbohydrate composition of sugarcane cultivars that are resistant or susceptible to *Sugarcane yellow leaf virus"*** Axel Lehrer, Shih-Long Yan, Blanca Fontaniella, **Abdelaleim ElSayed**, Ewald Komor*

Axel Lehrer did grading of symptoms and carbohydrate determination.

Shih-Long Yan and Blanca Fontaniella did the *In situ* determination of starch.

I have designed the specific primers to detect the SCYLV and determined the viral infection.

Ewald Komor supervised this work and was involved in scientific discussions. The publication was written jointly with Ewald Komor.

Chapter 5

This work is published in ***European Journal of Plant Pathology*, 2010, 127: 207-217,** under the title, **"Sugarcane yellow leaf virus introduction and spread in Hawaiian sugarcane industry: Retrospective epidemiological study of an unnoticed, mostly asymptomatic plant disease"** Ewald Komor*, **Abdelaleim ElSayed**, and Axel T Lehrer

Ewald Komor did the search for SCYLV in plantations in Hawaii and worldwide. He did the determination of distances, supervised this work and was involved in scientific discussions and suggestions.

I have detected the sugarcane yellow leaf virus by RT-PCR and Northern Blot analysis.

Axel T Lehrer did the determination of distances for de novo infection with SCYLV.

The manuscript was written jointly with Ewald Komor.

Chapter 6

This work is submitted to ***European Journal of Plant Pathology***, under the title, **"Molecular characterization of Hawaiian Sugarcane yellow leaf virus (SCYLV) genotypes and their**

phylogenetic relationship to SCYLV-strains from other sugarcane-growing countries" Abdelaleim ElSayed, Alfons Weig and Ewald Komor*

I have done all experiments and characterized all the presented data here except the cleaning of sequences. The manuscript was written by me.

Alfons Weig did cleaning of the sequences.

Ewald Komor supervised this work and was involved in scientific discussions, suggestions and correction of the manuscript.

Chapter 7

This work is submitted to ***Physiological and Molecular Plant Pathology*** under the title, **"Expression of sucrose transporter (ShSUT1) in a Hawaiian sugarcane cultivar infected with Sugarcane yellow leaf virus (SCYLV)" Abdelaleim Elsayed**, Mohamed Fawzy Ramadan and Ewald Komor*

I have done all experiments and characterized all the presented data here except the determination of sugar content. The manuscript was written by me.

Mohamed Fawzy Ramadan did the determination of sugar content.

Ewald Komor supervised this work and was involved in scientific discussions, suggestions and correction of the manuscript.

Chapter 8

This work is submitted to ***Plant Pathology*** under the title, **"Simultaneous quantitative analysis of transcripts for Sugarcane yellow leaf virus, sucrose transporters and sucrose phosphate synthase in Hawaiian sugarcane cultivars by multiplex RT-PCR" Abdelaleim ElSayed**, Alfons Weig and Ewald Komor*

I have prepared the plant samples, all the presented data in this work have been characterized by me. The manuscript was written by me.

Alfons Weig provided the GenomeLab Genetic Analysis System (GeXP) and was involved in correction of the manuscript.

Ewald Komor supervised this work and was involved in scientific discussions, suggestions and correction of the manuscript.

J Gen Plant Pathol (2010) 76:62–68
DOI 10.1007/s10327-009-0210-0

VIRAL AND VIROID DISEASES

Carbohydrate composition of sugarcane cultivars that are resistant or susceptible to *Sugarcane yellow leaf virus*

Axel Lehrer · Shih-Long Yan · Blanca Fontaniella · Abdelaleim ElSayed · Ewald Komor

Received: 28 August 2009 / Accepted: 8 October 2009 / Published online: 3 December 2009

Abstract Sugarcane cultivars with a high (susceptible cultivars) and low (resistant cultivars) virus titer of *Sugarcane yellow leaf virus* were grown in the field. The carbohydrate composition in green leaf tops and in stems was determined. In RT-PCR of leaf extracts, susceptible cultivars had a high SCYLV-titer, whereas resistant cultivars had a very low titer. The cultivars differed in biomass yield, but these differences were not correlated with susceptibility. However, carbohydrate composition did have susceptibility-specific differences. Hexose levels were lower in green leaf tops and stalks of susceptible (strongly infected) cultivars than in those of resistant (weakly infected) cultivars. The stalks of susceptible cultivars also had less starch than those of resistant cultivars. Thus, the viral susceptibility (and infection) affected sugar metabolism. In addition, a positive correlation between hexose and starch in stems and between hexose and sucrose in green leaf tops was observed. The results from susceptible versus resistant cultivars were the opposite of those in the comparison between infected versus virus-free lines of the same cultivar. The breeding process apparently had unintentionally selected clones with modulated carbohydrate metabolism to avoid or compensate for the adverse effects of SCYLV infection.

Keywords Biomass · Hexoses · *Saccharum* species · SCYLV · Starch · Sucrose · Yellow leaf

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Introduction

Yellow leaf (YL) was noticed as a sugarcane disease in the 1990s when leaf yellowing and a yield decrease were reported from plantations in different regions of the world (Comstock et al. 1994; Schenck 1990). The polerovirus *Sugarcane yellow leaf virus* (SCYLV) was identified as the responsible pathogen (Vega et al. 1997). A survey of sugarcane cultivars in Hawaii revealed that some cultivars had a high SCYLV titer, whereas others had little or no virus (Schenck and Lehrer 2000). The cultivars with the high titer were considered as susceptible, the others as resistant. In studies of the pathogenicity, infected plants maintained the virus in stem pieces and rootstocks (Lehrer et al. 2007; Rassaby et al. 2003). Virus-free plants of susceptible cultivar H87-4094 were generated by meristem tip culture (Fitch et al. 2001), and these virus-free plants had 10–30% higher yield than infected plants (Lehrer et al. 2009). However, when different cultivars were compared, SCYLV-susceptible and -resistant cultivars (containing the "natural" SCYLV titer) did not differ significantly in growth or yield (Lehrer et al. 2009). The screening process for the breeding program was thus concluded to have selected for clones that had compensated for the adverse effects of SCYLV. In a carbohydrate analysis of infected

plants, SCYLV impeded the export of assimilate from leaves (Lehrer et al. 2007; Yan et al. 2009). As in the previous comparison of infected and virus-free plants of the same cultivar, we undertook a similar analysis to compare susceptible and resistant cultivars (which contained, respectively, a naturally high and low virus titer) to reveal possible adaptations of the cultivars to cope with SCYLV infection.

Materials and methods

Growth and harvest of plants

Planting followed plantation practices. Stem cuttings 50 cm long and containing three nodes were incubated in water at 50°C for 30 min, then dipped in a propiconazole solution (Tilt, 25 g a.i./l, Novartis, Basel, Switzerland). Three field plots at the Experiment Station of Hawaii Agriculture Research Center (HARC) in Kunia were planted with 16 seed pieces of each cultivar in two parallel rows 1 m apart and 3 m long, with a drip irrigation tube in the centre covered with soil. The test plots were treated identically to commercial field practice.

The following cultivars were selected: susceptible cvs. H73-6110, H78-3606 and H87-4094, moderately susceptible cvs. H65-7052 and H78-3567, and resistant cvs. H78-4153, H78-7750, H87-4319 and H82-3569. All cultivars contained SCYLV; however, the titers were high in the susceptible and low in the resistant cultivars. A virus-free line of H87-4094 was generated by meristem tip culture (Fitch et al. 2001) and compared with the infected line of this cultivar.

The sugarcane was harvested after 16 months according to standards of the Hawaiian Breeding Program. All stems in 1.5 m of one double row, i.e., stems from eight stools, were cut at soil level. The green leaf tops were removed from the stalks, and tops and stems were weighed and chopped separately. One-kilogram samples of the chopped material were immediately frozen for later sugar extraction and carbohydrate analysis. Thus in each of the three plots, eight plants of each cultivar were harvested, weighed and chopped together. Sugar was determined in samples from that chopped, mixed material. Sugar was extracted from the chopped plant material as described by Payne (1968). One kilogram of chopped plant material was suspended in 2 l hot water for 15 min in a disintegrator, then the suspension was centrifuged, and the supernatant (=extract) was used for sugar determination.

Carbohydrate determination

Hexose determination Extract (0.2 ml) and ethanol (0.2 ml) were mixed and centrifuged for 10 min at 14,000 rpm, then 20 µl of this supernatant were added to 180 µl reaction buffer (100 mM triethanolamine–Cl pH 7.6, 3 mM NADP, 5 mM ATP, 10 mM $MgSO_4$). The enzymatic reactions were started by addition of 0.3 U hexokinase, 0.15 U glucose-6-phosphate dehydrogenase, and 1 U phosphoglucose isomerase. The products were measured after a 30-min reaction in a microplate reader at 340 nm.

Sucrose determination Extract (20 µl) was treated with 1.38 ml KOH (7.5%) at 90°C for 10 min. Then 140 µl of this reaction product were incubated for 20 min with 1 ml anthron reagent (150 mg anthron + 76 ml sulfuric acid + 30 ml water) at 37°C. An aliquot (200 µl) of the sample was measured in a microplate reader at 630 nm.

Starch determination The pellet after sugar extraction was washed, suspended in 200 µl and heated for 5 min in hot water. Then 2.5 µl α-amylase solution (STA-Kit, Sigma-Aldrich, St. Louis, MO, USA) and 200 µl STA2 reagent (10 U amyloglucosidase) were added, and the mixture was incubated for 15 min at 60°C. Samples of 10–50 µl of each reaction mixture were assayed for hexoses as described.

In situ determination of starch

Pieces of leaves were taken from the top visible dew lap leaf, sampled shortly before sunrise, and frozen in liquid nitrogen. The tissue samples were dehydrated in an ethanol series, embedded in paraffin, cross-sectioned (10 µm thick) with a microtome and rehydrated in an ethanol series, exactly as used for in situ hybridization (Woo et al. 1999). The sections were fixed on microscope slides and incubated in iodine solution (1 g KI and 1 g iodine in 100 ml water) for 10 min, then stopped with water for 5 s. The water was wiped off, the slides were air-dried, and 200 µl of Aquatex mounting medium (Merck, Darmstadt, Germany) was added to the slides before covering with cover slips. The cross-sectional area of the stained starch granules and of the cells was determined with Image-Pro software (Media Cybernetics, Bethesda, MD, USA).

Grading of symptoms

Symptoms were visually recorded at 6–8-week intervals and graded from 0 (no symptoms) to 6 (very severe symptoms) as described in Lehrer and Komor (2008).

Determination of viral infection

Source leaves of different cultivars were homogenized, the RNA was purified from the soluble extract with phenol–chloroform and transcribed to cDNA (Comstock et al. 1998; Sambrook and Russell 2001). The cDNA was

amplified by RT-PCR with gene-specific primers (forward: 5′-CTTTCAAGGTTCGCTCGTTC-3′, reverse: 5′-TGAGC TGGTTGACTGGAGTG-3′) creating a 165-bp fragment.

The presence of SCYLV was also tested by tissue blot immunoassay (TBIA) as described previously (Fitch et al. 2001).

Statistics

The significance of any differences was calculated by the program SigmaStat3.1 (Systat Software, Richmond, CA, USA), using a pairwise *t*-test, pairwise Mann–Whitney test, or a Kruskal–Wallis one-way analysis of variance on ranks for multigroup data. Regression lines and r^2 were calculated by the statistics function of the program SigmaPlot9.0 (Systat).

Results

Selection of susceptible and resistant cultivars

A previous survey using a TBIA had identified SCYLV-susceptible and SCYLV-resistant cultivars (Lehrer et al. 2009; Schenck and Lehrer 2000). Cultivars that expressed fluctuating levels of virus titer were called moderately susceptible. Tests for SCYLV by RT-PCR partly confirmed the different titers of virus in the different cultivars; however, the so-called resistant cultivars (H78-4153, H87-4319, H78-7750) that had appeared without SCYLV in TBIA, had SCYLV (Fig. 1), though at a much lower titer than, for example, H87-4094. H65-7052 appeared nearly

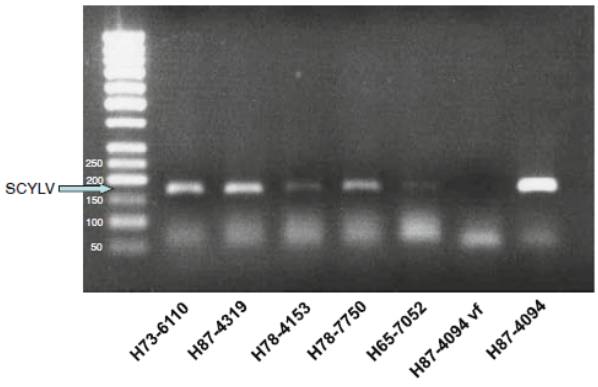

Fig. 1 RT-PCR of RNA-derived cDNA from source leaves of different cultivars of sugarcane. RNA from leaves of six representative cultivars was extracted, transcribed to cDNA and amplified by RT-PCR. H73-6110 and H87-4094 are susceptible, H65-7052 is moderately susceptible, and H78-4153, H87-4319 and H78-7750 are resistant cultivars. The virus-free clone of H87-4094 was used as a negative control. The amplified *Sugarcane yellow leaf virus* (SCYLV) was 165 bp long, the size standard was a 50-bp DNA ladder (*left*)

virus-free, possibly the leaf had been sampled in a virus-poor phase. Nine cultivars were selected for a test of carbohydrate status of sugarcane plants at harvest time (16 months), the same cultivars that had been used previously in an extended yield test in different Hawaiian fields (Lehrer et al. 2009).

Biomass of green leaf tops and stalks

The cultivars were grown in three plots in the field station of HARC and harvested after 16 months. The plants were cut by hand, and green leaf tops were separated from their stems. The stalk biomass of the different cultivars ranged between 100 and 200 t/ha, depending on cultivar and plot. The biomass of green leaf tops ranged between 15 and 45 t/ha. Differences showed up between some cultivars, but there was no correlation with SCYLV susceptibility (Fig. 2a, b). Severely symptomatic SCYLV-infected plants had bushy leaf tops, and differences might have been expected in the ratio of stalks to leaf tops. Visual inspections during the 4 months before harvest indeed showed that the high-titer, susceptible cultivars had mild to moderately severe symptoms in contrast to the low-titer, resistant cultivars (Table 1). However the stalk to leaf top ratio was not correlated with SCYLV susceptibility (Fig. 2c).

Carbohydrate content of green leaf tops and stalks of SCYLV-susceptible and SCYLV-resistant cultivars

In situ localization and determination of starch in source leaves, harvested shortly before sunrise, showed that the susceptible cultivars had more starch in the bundle sheath cells than did the resistant cultivars, but even the resistant cultivar had higher starch levels than in the leaves of the virus-free plant (Fig. 3). The ratio of starch to cytoplasm area was significantly lower ($P < 0.05$) in the virus-free compared to the resistant and the susceptible cultivars (Table 2). The susceptible cultivar even had small starch grains in the mesophyll, a feature that was absent in the virus-free and the resistant cultivars.

When the plants were harvested during the day, the carbohydrates of complete green leaf tops consisted mostly of sucrose, which was 4-fold higher (when based on hexose units) than the hexose content and 100-fold higher than the starch content (Table 3). The sucrose and the hexose levels were not significantly different between resistant and susceptible cultivars, starch had a slight trend toward lower content in the susceptible cultivars ($P = 0.19$, Table 3). The stalks contained (as expected) mostly sucrose. The sucrose content was 40-fold higher (based on hexose units) than the content of hexoses and 200-fold higher than starch (Table 3). Susceptible cultivars had significantly lower

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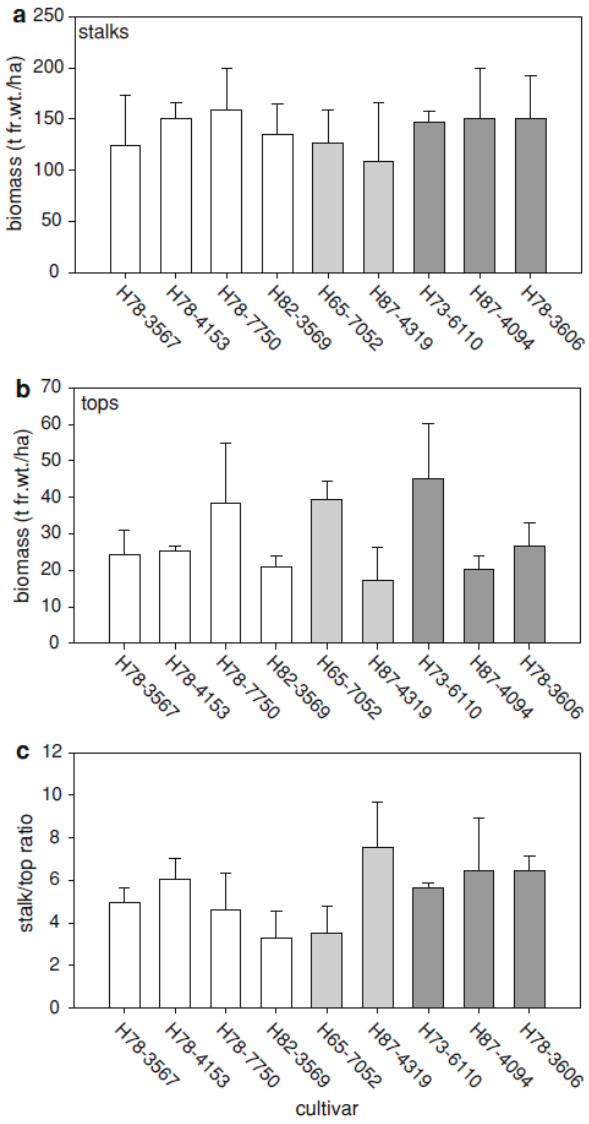

Fig. 2 Biomass (fresh mass) of stalks (**a**) and green leaf tops (**b**), and the ratio of stalk to green leaf tops (**c**) of *Sugarcane yellow leaf virus* (SCYLV)-resistant and -susceptible cultivars infected by SCYLV. SCYLV-resistant cultivars (*white*), moderately susceptible cultivars (*grey*), susceptible cultivars (*dark grey*). Mean ± SD

Table 1 Symptom expression of cultivars in field Kunia during the last 4 months before harvest, either 350 or 450 days after planting

Cultivar (R, titer levels)	Symptom index[a]	
	350 days	450 days
H78-3567 (R, low)	0	0
H78-4153 (R, low)	0	0
H78-7750 (R, low)	0	0
H82-3569 (R, low)	0	0
H65-7052 (moderately S, fluctuating high)	2	3
H87-4319 (moderately S, fluctuating low)	0	0
H73-6110 (S, high)	4	1
H87-4094 (S, high)	4	1
H78-3606 (S, high)	1	0

R resistant, *S* susceptible

[a] Symptoms were indexed visually as described by Lehrer and Komor (2008) at 1–2 months intervals

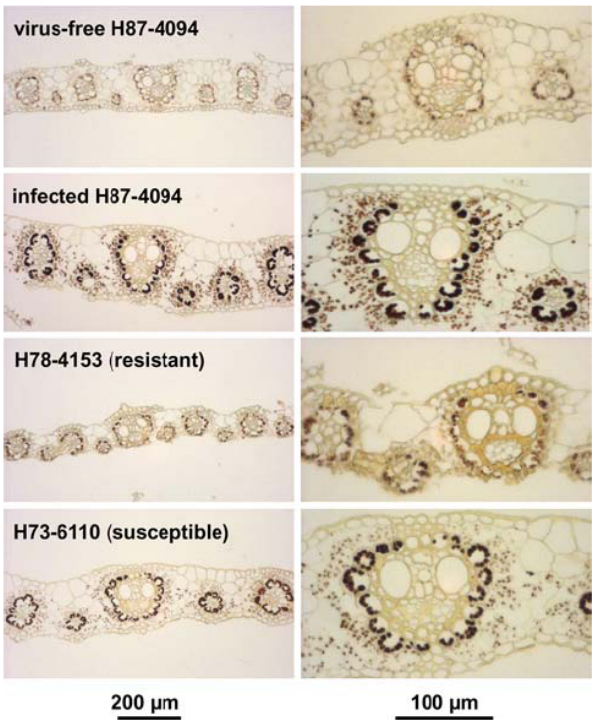

Fig. 3 Starch in sugarcane leaves from *Sugarcane yellow leaf virus* (SCYLV)-free, SCYLV-resistant and a SCYLV-susceptible (high titer) cultivar. Leaf samples from SCYLV-free and SCYLV-infected plant of cv. H87-4094, from the SCYLV-resistant cv. H78-4153, and from the susceptible (infected) cv. H73-6110 were collected in the hour shortly before sunrise, fixed, sectioned and stained with iodine

hexoses and starch in the stalks than did the resistant cultivars (Table 3). The moderately susceptible cultivar H65-7052 with strongly fluctuating SCYLV-titer was grouped with the susceptible cultivars, because it contained a 100-fold higher SCYLV-titer than in the other moderately susceptible cultivar H87-4319 (J. Zhu et al., unpublished manuscript). In contrast H87-4319 was grouped with the resistant cultivars based on its virus titer.

The carbohydrate species had some correlation among each other. In the green leaf tops, hexoses and sucrose were positively correlated, hexose and starch were negatively correlated (Fig. 4a). In stalks, hexoses were correlated positively with starch and slightly negatively with sucrose (Fig. 4b).

Table 2 Mean ratio of starch to cytoplasm in bundle sheath cells of source leaves in cultivars free of *Sugarcane yellow leaf virus* (virus-free), high-titer (susceptible) and low-titer (resistant) cultivars

Cultivar (resistance, titer)	*N*	Ratio ± SD
H87-4094 (S, virus-free)	65	0.14 ± 0.06^{a}
H87-4094 (S, high)	25	0.41 ± 0.10^{c}
H78-4153 (R, low)	46	0.25 ± 0.09^{b}
H73-6110 (S, high)	75	0.37 ± 0.12^{c}

Ratios were measured using Image-Pro software

Different letters after the mean ratios indicate significant differences between groups at $P < 0.05$, Kruskal–Wallis one way analysis of variance on ranks

Table 3 Mean levels of sucrose, hexoses and starch in green leaf tops and stalks of *Sugarcane yellow leaf virus* (SCYLV)-resistant (low titer) and -susceptible (high titer) sugarcane cultivars

Plant part	Resistant cultivars	Susceptible cultivars
Green leaf tops		
Hexoses (µmol/g fresh mass)	26.3 ± 5.3	23.4 ± 4.5^{ns}
Sucrose (µmol/g fresh mass)	52.6 ± 18.3	58.2 ± 20.6^{ns}
Starch (µmol hexose/g fresh mass)	1.59 ± 0.43	$1.10 \pm 0.31^{(*)}$
Stems		
Hexoses (µmol/g fresh mass)	7.52 ± 1.40	5.09 ± 1.80*
Sucrose (µmol/g fresh mass)	159.1 ± 4.5	164.4 ± 11.8^{ns}
Starch (µmol hexose/g fresh mass	1.26 ± 0.38	0.47 ± 0.25**

Values for resistant cultivars (H78-4153, H78-7750, H87-4319, H82-3569 and H78-3567) and for susceptible cultivars (H73-6110, H78-3606 and H87-4094 and H65-7052) were summed. The moderately susceptible cultivar H65-7052 with strongly fluctuating SCYLV titer was grouped with the susceptible cultivars, because it contained a 100-fold higher SCYLV titer than did the other moderately susceptible cultivar H87-4319 (J. Zhu et al., unpublished manuscript). In contrast, H87-4319 was grouped with the resistant cultivars based on its virus titer

ns Not significant

** $P < 0.05$, * $P < 0.10$, (*) $P = 0.10$–0.20

Discussion

Infection of sugarcane plants by SCYLV causes 10–30% yield reduction even when the plants are asymptomatic. However, the yields of naturally infected (the strain was probably the BRA-PER type; A. ElSayed, personal observation) cultivars of different SCYLV-susceptibilities, did not differ in tests in 10 Hawaiian fields (Lehrer et al. 2009). In general, this picture also emerged in the study here, where biomass of susceptible and resistant cultivars was compared in more detail, namely, of green leaf tops and of stalks, grown in a field of the Experiment Station. There were some differences in biomass between the cultivars on this particular field, but obviously, the difference was not along SCYLV-susceptibility.

The differences in carbohydrate composition indicated that SCYLV susceptibility had an impact on carbohydrate physiology of the plants. In a comparison of carbohydrate partitioning in SCYLV-infected and virus-free plants of cv. H87-4094, sucrose export from the leaves was reduced in the infected plants (Lehrer et al. 2007), resulting in a backup of sucrose, changes in chloroplast ultrastructure and finally degradation of chlorophyll (Yan et al. 2009). The present study showed that leaves of a susceptible cultivar harvested at the end of the night had stronger starch staining than did the resistant cultivar (Fig. 3), similar to the results of Yan et al. (2009). However the green leaf tops of susceptible cultivars when harvested during daytime revealed a trend toward less starch (Table 2). This discrepancy can be explained in two ways. The higher starch content in infected leaves is only evident at the end of the night when normally all transitory starch has been degraded and mobilized, whereas during day the starch levels rise strongly, and differences in starch are obscured. In addition, the green leaf top contains not only green source leaves, but also rolled-in sink leaves and the apical division zone internodes. Thus, the carbohydrate composition of the green leaf top is dominated by the young internodes and sink leaves, in which the resistant cultivars apparently contain more starch than the susceptible cultivars. Hexose in the leaves originates from transitory starch breakdown, which may explain rising hexose at the cost of starch.

No differences were observed in sucrose content of the green leaf tops, in contrast to SCYLV-infected plants of the cultivar H87-4094, where the sucrose concentration in the green leaf tops was 1.93-fold (±0.32, SD) higher than in virus-free plants. Hexose is positively correlated to sucrose. An increase in hexoses may result in more sucrose when sucrose is in equilibrium with hexose via sucrose synthase.

The stems of susceptible cultivars had less hexoses and less starch than resistant cultivars (Table 3), which was similarly observed in infected versus virus-free plants of cv. H87-4094 (Lehrer et al. 2007). In general, lower hexose and starch content indicates a more mature state of sugarcane internodes. So it appears that stem internodes of SCYLV-susceptible cultivars were faster in ripening than resistant cultivars, a feature that is observed to the extreme when sugarcane plants became symptomatic (Lehrer et al. 2007). Sucrose is delivered to the stem, and hexose is produced by invertase; thus high hexose content favors starch synthesis.

In conclusion, the SCYLV-susceptible cultivars differed from the resistant cultivars in carbohydrate composition, indicating an impact of SCYLV on sugar physiology. However, some results, for example, the biomass ratio of stalk to green leaf tops and the amounts of sucrose and

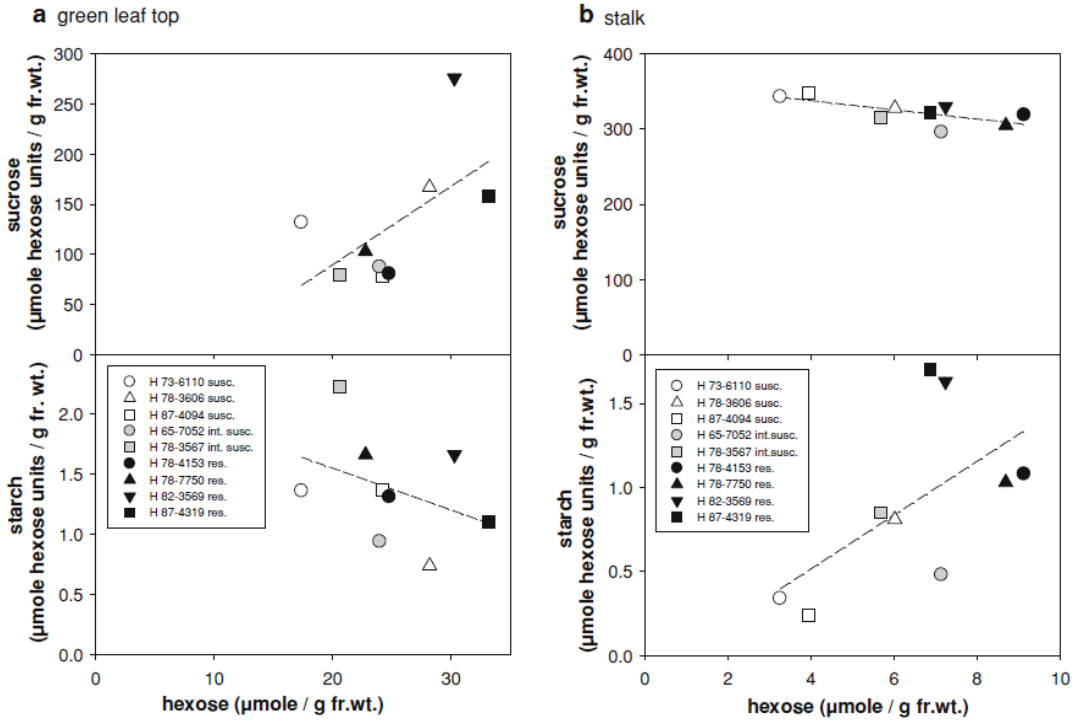

Fig. 4 Relationship between the hexoses, sucrose and starch content per gram fresh mass of sugarcane green leaf tops and stems at harvest. The plants were harvested at noon at the age of 16 months. *Open symbols* susceptible (high titer) cultivars, *grey symbols* moderately susceptible (moderately high titer) cultivars, *black symbols Sugarcane yellow leaf virus*-resistant (infected but with low titer) cultivars. Data are the mean for eight plants in three replicates. The regressions of the plots for green leaf tops, upper graph $r^2 = 0.88$, lower graph $r^2 = 0.93$, for stems (*right*) upper graph $r^2 = 0.99$, and lower graph $r^2 = 0.82$

starch in green leaf tops were opposite of the results obtained for cv. H87-4094 when comparing infected to virus-free plants (Lehrer et al. 2007). That discrepancy may lie in the process by which commercial cultivars are selected. The breeding program selected cultivars for maximum yield under the Hawaiian field conditions in the presence of SCYLV, so that lower-yielding clones of the progeny were eliminated, irrespective of a latent SCYLV infection. Therefore SCYLV-susceptible clones had to compensate by other means for the virus-caused sucrose backup, possibly by increasing sucrose transport activities or decreasing sucrose retrieval along the translocation pathway. The SCYLV test by RT-PCR showed that all cultivars contained the virus but at very different levels; the virus was barely detectable in the resistant varieties. Quantitative RT-PCR revealed that the SCYLV titer differed by 2–3 orders of magnitude (J. Zhu et al., submitted).

This study underlines the ability of plants, exemplified here by different cultivars, to modulate their carbohydrate metabolism to avoid or compensate for the adverse metabolic effects caused by viral infection.

Acknowledgments The authors are grateful to the field workers of the Hawaii Agriculture Research Center for their help and to the staff of the analytical lab for standard sugar determinations. Thanks to Alberto Bertolini, University Udine, who participated in the in situ starch determination project. This work was funded by Deutsche Forschungsgemeinschaft and by Deutscher Akademischer Austauchdienst, which supported Yan Shih-Long, Alberto Bertolini and Blanca Fontaniella.

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Eur J Plant Pathol (2010) 127:207–217
DOI 10.1007/s10658-010-9585-1

Sugarcane yellow leaf virus introduction and spread in Hawaiian sugarcane industry: Retrospective epidemiological study of an unnoticed, mostly asymptomatic plant disease

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Accepted: 14 January 2010 / Published online: 8 February 2010

Abstract Yellow leaf (YL) caused by *Sugarcane yellow leaf virus* (SCYLV) was first reported as a sugarcane disease in the 1990s, when it had already spread over many parts of the world. The time of introduction into the plantations is unknown. A worldwide screening identified only a few places isolated from cultivar exchange for more than 20 years which appeared SCYLV-free. Control tests with infected cultivars propagated for 12–16 generations by cuttings remained SCYLV-infected, proving that SCYLV is not eliminated by vegetative propagation. *De novo* infection by SCYLV-vectors in Hawaii occurred only over short distances. To reveal the period when SCYLV was introduced to Hawaii, volunteer sugarcane plants from closed Hawaiian plantations and from previous sites of the Hawaiian Sugarcane Planters' Association breeding station were tested. The results suggest that SCYLV appeared in the breeding station between 1960 and 1970, whereas the plantations became infested after 1980. Imports in the 1960s obviously introduced the virus to the Hawaiian breeding station from where it spread to susceptible cultivars. Eighty percent of the cultivars, developed between 1973 and 1995, acquired the virus at the breeding station, in some cases within 4 years, indicating the rapid spread of SCYLV in the breeding station. The strain of SCYLV found in a Réunion cultivar in Hawaii, and the differing SCYLV-infection of CP-cultivars which were exported more than 20 years ago, suggested that also Réunion and Florida may still have been SCYLV-free in the 1970s. The study showed that retrospective epidemiology can be conducted on a disease which was unnoticed for more than 20 years.

Keywords Sugarcane *Saccharum spec.* · Volunteer plants · Yellow leaf (YL)

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Introduction

The sugarcane disease yellow leaf (YL) was first detected in the early 1990s in Hawaii (Schenck 1990). Later, similar symptoms were reported from mainland United States (Comstock et al. 1994), Brazil (Vega et al. 1997), Mauritius (Moutia and Saumtally 1999) and many other sugarcane countries (Bailey et al. 1996; Victoria et al. 1998; Lockhart and Cronje 2000). The polerovirus *Sugarcane yellow leaf virus* (SCYLV) was identified as a possible causal agent (Vega et al. 1997). The virus arose as a recombination product of *Barley yellow dwarf virus* and *Potato leaf roll virus*

(Smith et al. 2000; Moonan et al. 2000) and 3–4 clusters of strains could be grouped, whereby a Colombian strain was proposed as the original population, which diverged out of the polerovirus group (Moonan and Mirkov 2002; Abu Ahmad et al. 2006). The fact that the disease was detected not earlier than in the 1990s points to a more recent worldwide distribution, even though a report in 1968 about yellow wilt in sugarcane in Eastern Africa is attributed as, possibly, a first account of yellow leaf (Ricaud 1968; Bailey et al. 1996). On the other hand, since YL and SCYLV respectively were detected at the same time in many countries in the late 1990s, a worldwide distribution of infected sugarcane must have occurred some time before the first recognition of the disease. The time of SCYLV introduction into the plantations is unknown, because the infection remains mostly asymptomatic and therefore unnoticed. Specific tests for SCYLV have been available only since the late 1990s (Schenck et al. 1997; Comstock et al. 1998).

The sugar industry in Hawaii was founded in the mid-1850s and had expanded up to World War II with more than 50 sugarcane estates on five islands. By merger and closure the number was reduced to 10 in the 1990s and currently (2009) to two. Although many former sugarcane fields were transferred to other crops, still some sugarcane plants survived over many years as volunteers due to the favourable, tropical climate and the fact that sugarcane is a perennial plant. These volunteer plants might represent a testimony of the SCYLV-infestation state of the plantations at the time when they were no longer used for sugarcane cultivation. The idea of the following study was to search and test these volunteer plants from the former plantations for SCYLV with the aim to identify a time range in which the Hawaiian plantations became infested by SCYLV.

Material and methods

Growth of plants

Plants grown in the field in Hawaii were planted and cultivated following general cultural practices. Seed pieces of approximately 50 cm length (containing 3 nodes) were cut from a virus-free seed plot in Laie, Oahu. They were hot water treated at 50°C for 30 min and then dipped in fungicide propiconazole at 25 g a.i./l (Tilt, Novartis).

Plants in pots were grown in the greenhouse at Bayreuth University from single-node seed pieces. The seed pieces were treated the same way as described above. They were first germinated on wet paper towels and, when the shoots and roots had developed, the seedlings were planted in 20 l pots with commercial potting soil. The potted cultivars were routinely replanted twice a year with stem cuttings.

Virus-free bait plants of the highly susceptible cultivar H87-4094 were grown in the greenhouse as bait plants to prove that the greenhouse is free of SCYLV insect vectors.

Determination of distances for de novo infection with SCYLV

The short-distance spread of SCYLV-infection between plants was determined with virus-free, susceptible plants of cultivar H87-4094. They had been generated by meristem tip culture as previously described (Fitch et al. 2001). The seedcane plot for these virus-free plants was at Brigham Young University garden at Laie, Oahu, remote from active sugarcane research or plantation fields.

For the determination of very short-distance spread (1–3 m), virus-free plants were planted in test plots in fields, next to SCYLV-infected plants. The seed pieces were planted in parallel rows at 1 m spacing with eight seed pieces per row. Fertilization and irrigation was made according to plantation practice. For determination of infection spread over longer distances, virus-free plants were planted in small plots with at least 5 seed pieces at selected places distant from infected, operating sugarcane plantations. In three cases virus-free, susceptible plants from former plantations were collected in previous, now abandoned sugarcane fields.

Collection of leaf samples

The fields of former sugarcane plantations were surveyed for volunteers in the years 2001 and 2003. Leaf samples were collected from the sugarcane plants and preserved for the day in plastic bags. On the same day tissue prints of the leaf midribs were made on nitrocellulose membranes. The leaf samples from plants in the breeding station at Maunawili were collected in the year 2000 and processed the same way.

The seed pieces for PCR and northern Blot were obtained from plants in the greenhouse of Bayreuth

University. The detection of SCYLV in newly emerged seedlings was performed from freshly cut seed pieces germinated in an insect-tight cage for 3 weeks.

Tissue prints were also produced mostly in the years 2000–2002 by researchers in India (G.P. Rao), Phillippines (R. Cu and F. dela Cueva), Guadeloupe (J.-H. Daugrois), Morocco (J. Enahari) and Spain (H. Richter) and sent to Hawaii Agriculture Research Center (HARC), Aiea, for further development, which was performed by Dr. S. Schenck. Leaf material and/or stem pieces of sugarcane were collected near Termez, Uzbekistan, from the Sugar Crops Research Institute, Giza, Egypt, and from private fields near Baniyas, Syria (Z. Soufi). This material was tested for SCYLV by tissue blot immunoassay (TBIA) and by RT-PCR.

Test for SCYLV by TBIA, RT-PCR and northern Blot

The membranes were kept in sealed Petri dishes until development for TBIA. They were incubated with antibody against SCYLV (a gift from Dr. Lockhart, University of Minnesota), then incubated with goat anti-rabbit alkaline phosphatase conjugate (Fitch et al. 2001). The colour development was observed under the microscope.

RNA was extracted from leaf samples and tested for SCYLV by RT-PCR and northern Blot (Comstock et al. 1998; Sambrock and Russell 2001). The freshly emerged leaves (about 10 cm high) of the germinated bud were homogenized and the RNA was purified from the soluble extract with phenol-chloroform. Twenty µg of RNA was separated on a 1.2% formaldehyde agarose gel, and RNA was transferred to a positively charged nylon membrane (0.45-µm pores, Hybond N^+, Amersham) by capillary transfer. The RNA probe to SCYLV was produced by using PCR generated templates for *in vitro* transcription. The DNA fragments were amplified by RT-PCR with gene-specific primers (FW: 5′-CTTTCAAGGTTCGCTCGTTC-3′, RV: 5′-TGAGCTGGTTGACTGGAGTG-3′) and cloned into pGEM®-T, creating a 165 bp fragment. The orientation of the insert fragment was determined by sequencing. The transcription of RNA anti-sense probe and the hybridization were performed as described in the DIG System Users Guide (Roche Diagnostics, Switzerland).

The test for SCYLV in different cultivars by RT-PCR was performed with RNA extracted from source leaves. The primers for PCR were the same as described above, producing a 165 bp fragment of SCYLV.

Plantation records

The business history of the Hawaiian sugarcane estates was investigated in the library of HARC Experiment Station, Aiea, (former Hawaiian Sugar Planters´ Association (HSPA)) and the Kauai Museum, Lihue. The sugarcane cultivars, which had been planted in the different plantations during their operation, were traced in the records of the breeding department of former HSPA.

Results

Worldwide distribution of SCYLV

The worldwide distribution of yellow leaf syndrome (YLS) was first reported by Lockhart and Cronje (2000) as a "disease of uncertain etiology". We collected samples from many sugarcane plantations around the world in the early 2000s and most of them proved to be infected by SCYLV (Fig. 1). When these results are complemented by reports from other research groups (references in Fig. 1), the worldwide dissemination of SCYLV is obvious. SCYLV-free samples were obtained from a few places only, namely Termez, Uzbekistan, where sugarcane cultivation had been terminated in 1988, Salobreña, Spain, where production ceased in 2002, farmer fields in Baniyas, Syria, where no new cultivars were introduced in the past 50 years, and cultivars from Giza, Egypt. Differing results were obtained for some old cultivars from Hawaii, Florida and Louisiana (Table 1); in some places these cultivars contained SCYLV, in others they were SCYLV-free. The fact that these cultivars were infected in some places indicated that they were susceptible to the virus, but were probably SCYLV-free at the time of export. In summary it appeared that sugarcane plantations, which were isolated from cultivar exchange in the past 20–30 years, and some old cultivars exported more than 20 years ago, were SCYLV-free.

Maintenance of SCYLV-infection in sugarcane stalks

Sugarcane is propagated vegetatively by cuttings. It was important to show whether SCYLV is

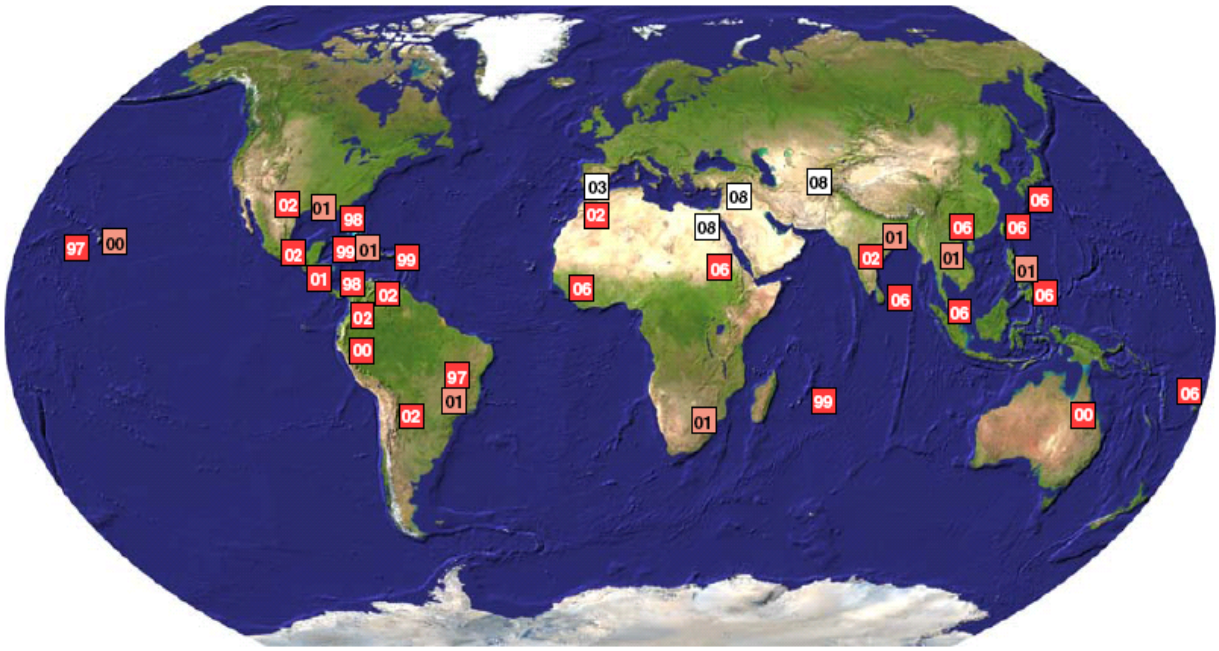

Fig. 1 *Proven presence of SCYLV in sugarcane regions of the world*. Squares in red indicate the presence of SCYLV and white squares the absence of SCYLV, light red according tests conducted by the authors, dark red according published reports (Schenck et al. 1997; Vega et al. 1997; Comstock et al. 1998; Victoria et al. 1998; Moutia and Saumtally 1999; Arocha et al. 1999; Alegria et al. 2000; Smith et al. 2000; Avila et al. 2001; Comstock et al. 2002; Nadif et al. 2002; Rassaby et al. 2004; Abu Ahmad et al. 2006). The numbers in the squares show the year, when the tests for SCYLV were made respectively were reported. (World map reproduced from www.uwec.edu and modified)

Table 1 SCYLV-infection state of some old cultivars in different areas of the world. The tests (TBIA) were conducted in 2000 and 2001

Cultivar	SCYLV-infection	Origin of collection
H39-3633	−	Thailand; Guadeloupe (France)
	+	Hawaii (USA)
CP61-39	−	Thailand, Guadeloupe (France)
	+	Florida (USA)
L62-96	−	Kenitra (Morocco)
	+	Louisiana (USA)
CP65-357	−	Spain
	+	Florida (USA)
CP66-346	−	Kenitra (Morocco)
	+	Gharb (Morocco)
CP70-321	−	Kenitra (Morocco)
	+	Gharb (Morocco)
CP84-1198	−	Phillippines
	+	Thailand, Guadeloupe (France)

propagated by seed pieces to successive generations. Rassaby et al. (2004) already reported that SCYLV-infection was maintained during regrowth after ratooning. Cultivars which were imported as one-node seed pieces from Hawaii in 2001, were grown in the greenhouse at the Bayreuth University. The plants were cut 1–2 times per year and each time regenerated from seed pieces. The cultivars, which were SCYLV-infected 8 years ago when collected in the field (Schenck and Lehrer 2000), still contained SCYLV after the 12–16 cycles of replanting in the greenhouse (Fig. 2). When seed pieces of infected plants were germinated in an insect-proof cage, the freshly emerged leaves already contained SCYLV (Fig. 3). Virus-free plants of the susceptible cultivar H87-4094 remained virus-free in the Bayreuth greenhouse even when held outside of insect-proof cages over several years (Fig. 2), which indicates that the greenhouse is free of SCYLV-vectors. The presence of SCYLV in the infected cultivars over so many replantings did not therefore originate from *de novo* infection in the greenhouse.

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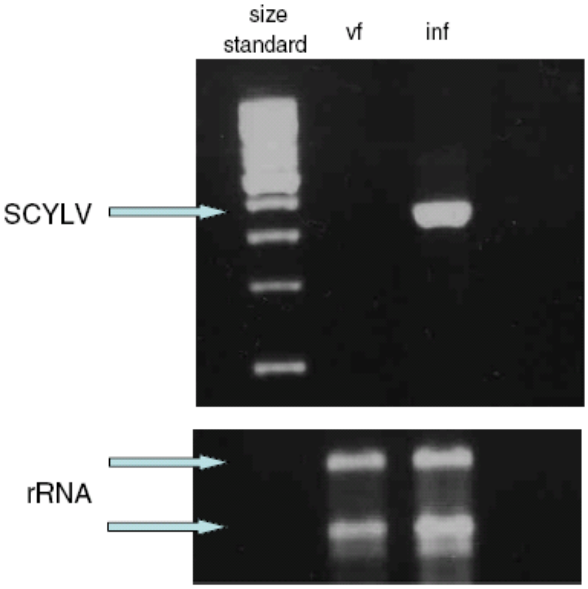

Fig. 2 RT-PCR for SCYLV in virus-free and infected cv. H87-4094 after 12–16 cycles of replanting, with rRNA as loading control. Virus-free (vf) and infected (inf) plants of H87-4094 were grown in the greenhouse outside of insect-proof cages. RNA from leaf samples was extracted and amplified with SCYLV-specific primers by RT-PCR. The reaction products were separated on gels and stained with ethidium bromide. Length of amplified SCYLV was 165 bp, the size standard was a 50 bp DNA-ruler from Fermentas (St. Leon Rot, Germany)

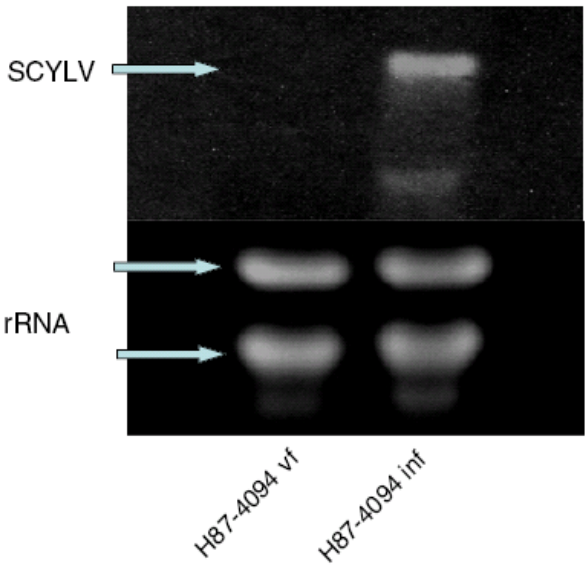

Fig. 3 Northern blot of RNA from freshly germinated seed pieces. Seed pieces of cv. H87-4094 were germinated for 3 weeks in insect-proof cages and RNA was extracted from the freshly emerged leaves. H87-4094 inf = infected cv. H87-4094, H87-4094 vf = virus-free plants of H87-4094. The RNA of SCYLV and, as a loading control, of rRNA is indicated by arrows

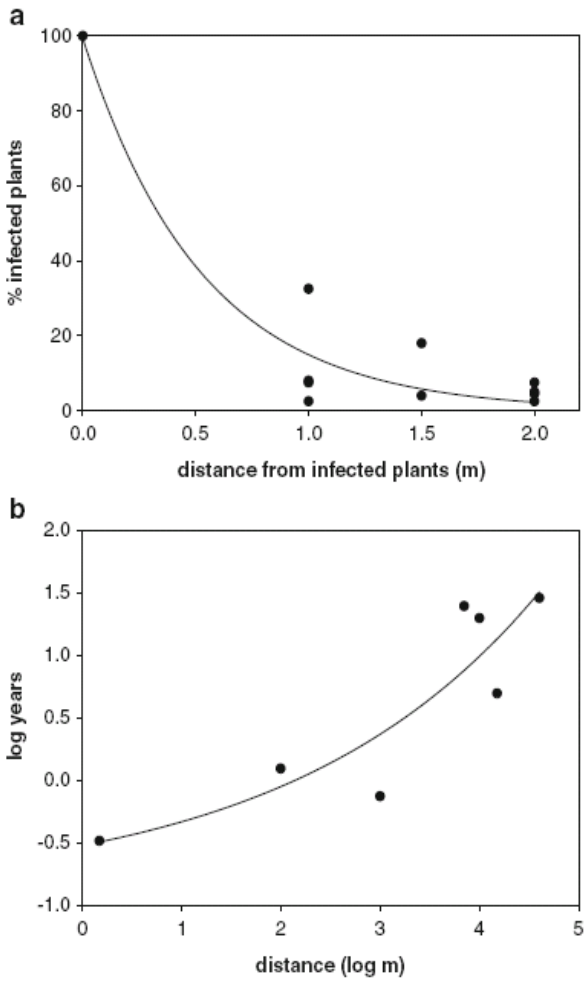

Fig. 4 De novo infection frequency of sugarcane plants placed at different distances. Virus-free, susceptible plants of cv. H87-4094 were grown at different distances from rows or fields of SCYLV-infected plants. The cultivar which remained virus-free for more than 20 years was H50-7209. a): Infection frequency at short distance after 11 months (r^2=0.93). b): Minimum time after which virus-free plants placed at different large distances were still virus-free. It represents the minimum time, because the plants might have stayed virus-free for longer (r^2=0.79)

Spread of SCYLV by de novo infection

The *de novo* infection in fields in Hawaii and in Louisiana by viruliferous aphids such as *Melanaphis sacchari* is relatively slow (Lehrer et al. 2007; McAllister et al. 2008). In controlled tests virus-free plants of the susceptible cultivar were planted in plantation fields at different distances from SCYLV-infected plants and tested for SCYLV after 11 months. On average only 5% of plants had acquired SCYLV at a distance of 2 m (Fig. 4a). Virus-free plants distant between 100 m and 25 km from infected plants were

not infected at the time of testing, which varied between 1 year and 30 years after planting (Fig. 4b). Apparently such dispersal distances are not likely for aphids in Hawaii.

SCYLV in sugarcane plants in fields of present and former plantations

Hundreds of samples had been collected from operating plantations in the late 1990s by Dr. Schenck, which led to the discovery of YL (Schenck 1990) and SCYLV (Schenck et al. 1997; Schenck and Lehrer 2000). This survey was extended in 2001 and 2003 to all plantations, which existed in Hawaii in the 1990s, and to fields of former plantations and estates, which went out of operation between 1945 and 1990. Leaf samples were collected, with each sample taken from a different stool. The number of samples was high from the operating plantations and from those which had closed recently (Table 2). In contrast only a few sugarcane clones were found in plantation fields that were abandoned more than 30 years ago, except the former estates in Kohala and in Kahuku. The sampled leaf pieces were tested for SCYLV by TBIA. Whenever one leaf sample reacted positively with the SCYLV-antibody, the plantation and site of collection were considered to be infested by SCYLV. The tests showed that all plantations which were still under operation or which were under operation up to recently, were infested, including plants from fields that were abandoned already in the 1980s (Fig. 5). In contrast, plantations from estates which went out of business before 1980 were SCYLV-free. This picture held true for each of the four "sugar islands" of Hawaii. As a reminder, the first yellowing symptoms were recorded in fields of two plantations (Hamakua and Oahu Sugar) in the late 1980s which led to the discovery of YL (Schenck 1990).

SCYLV in past breeding and quarantine station sites

The breeding station of HARC (former HSPA) was tested for SCYLV, including former sites of the breeding station and former quarantine station sites (Table 2). The breeding station had changed place several times. First it was placed what is now Lyon Arboretum, Honolulu. Volunteer plants at Lyon Arboretum were SCYLV-free. In 1960 the breeding station changed to Maunawili next to Pali highway. Volunteers from this site, which was closed 1972, were SCYLV-infected, which indicates that the breeding station had acquired SCYLV in the time between 1960 and 1972. A few clones were detected at the former quarantine station sites at Waianae (closed 1960) and Kaaawa (closed 1975). Whereas the plants from the former were virus-free, the plants of the latter were infected. All these findings narrow the time of SCYLV-introduction into the breeding station to the period of 1960–1972 (Fig. 5). Two further facts corroborate that conclusion: *Saccharum spontaneum* hybrid plants had been provided as windbreaks to papaya growers in 1975. These plants turned out to be infected with SCYLV. In 1982, seed pieces of cultivars H32-8560 and H50-7209 were exported to Peru and plants of these cultivars were later reported to be infected by SCYLV (Alegria et al. 2000). The cultivars may have acquired SCYLV in Peru, however, the authors reported (in 2000) that the plants exhibited unusual, severe leaf yellowing in the past 18 years, i. e. since 1982, when imported from Hawaii.

SCYLV spread in the present breeding station

Evidence from the former locations of the breeding station indicated that the breeding station had SCYLV at least 10 years ahead of the plantations. A survey was conducted, in which all newly-bred clones at the breeding station were tested for SCYLV to reveal whether infection of virus-free plants proceeds in the environment of the breeding station. On average 80% of the clones were infected, including those which had been derived from crossings just 4 years before (Fig. 6). Considering that all these clones originated from virus-free, true seeds, the plants must have acquired SCYLV in the breeding station through *de novo* infection by viruliferous aphids. Although the infection spread by aphids is relatively slow (Fig. 4a) it is obviously fast enough to infect the susceptible clones within a few years in the breeding station, where plants are standing at 1 m distances within and between rows. In the experiment 20% of plants at a distance of 1 m became infected within 11 months (Fig. 4a).

Sugarcane imports

It appears that the Hawaiian breeding station acquired SCYLV in the years between 1960 and 1970,

Table 2 Collection places and sample numbers of volunteer plants in recent and former Hawaiian sugarcane plantations. Only plantations which existed 1945 and later were considered. The company names listed here were those which existed at the time when the plantation was closed

Island, plantation name and place of sugar mill	year of plantation closure	number of collected samples
Kauai		
(1) Kilauea Sugar Co., Kilauea	1975	19
(2) McBride Sugar Co., Eleele	1986	11
(3) The Lihue Plantation Co., Anahola	1990	13
(4) Kekaha Sugar Co., Kekaha	1999	20
(5) The Lihue Plantation Co., Lihue	2000	>100
(6) Gay and Robinson, Kaumakani	in operation	>100
Oahu		
(1) Waianae Sugar Co., Waianae	1946	3
(2) Waimanalo Sugar Plantation	1947	3
(3) Honolulu Plantation Co., Honolulu	1950	3
(4) Laie Sugar Plantation, Laie	1953	6
(5) Kahuku Plantation Co., Kahuku	1972	32
(6) Oahu Sugar Co., Waipahu	1994	24
(7) Waialua Sugar Co., Waialua	1995	13
Maui		
(1) Kaeleku Sugar Co., Kaeleku	1946	7
(2) Olowalu Sugar Co. (some fields)	1982	3
(3) Pioneer Mill Co., Lahaina	1996	10
(4) Hawaiian Commercial and Sugar Co., Puunene and Paia	in operation	>100
Hawaii		
(1) Olaa Sugar Co., Kapoho	1960	23
(2) Kohala Sugar Co., Hawi	1973	48
(3) Puna Sugar Co., Olaa	1982	56
(4) Hamakua Sugar Co., Paauilo	1994	48
(5) Mauna Kea Sugar Co./Hilo Coast Processing Co., Wainaku, Pepeekeo	1994	58
(6) Kau Sugar Co. Pahala	1996	34
HSPA		
(1) Breeding station, Lyon Arboretum	1960	7
(2) Quarantine station, Waianae	1960	2
(3) Breeding station, Maunawili Pali Rd	1973	16
(4) Quarantine station, Kaaawa	1975	21
(5) Windbreaks for papaya farmers, windward Oahu	1975	3
(6) Export to Peru	1982	unknown
(7) Breeding station, Maunawili hill side	In operation	>100

probably by import of infected clones. The station imported 234 cultivars between 1955 and 1972, mostly from other sugarcane breeding stations worldwide, but also from a collection expedition to New Guinea. In the probably crucial period of 1960–1970 the majority of imports came from Asia and the Australo/Pacific area, but also some from North and South America (none from Colombia), and only few from Africa (Fig. 7). Thus no definitive hint as to the origin of an imported SCYLV-infected cultivar could be obtained from the import records.

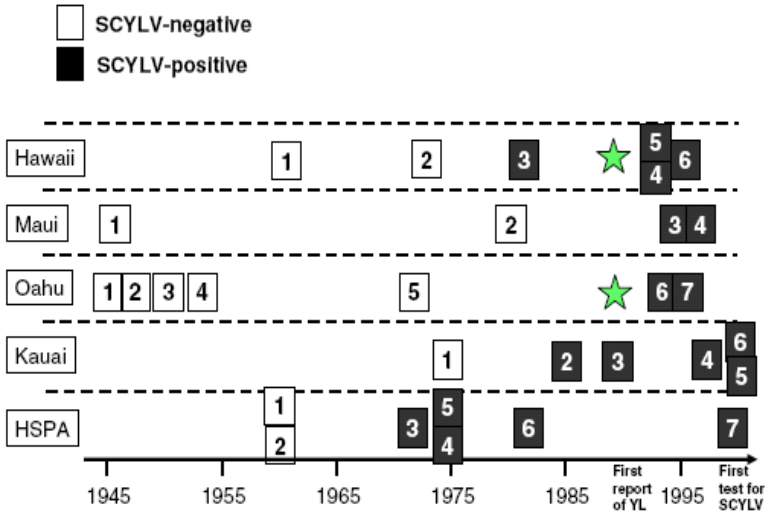

Fig. 5 SCYLV-infestation of past and present sugarcane plantations in Hawaii. Sugarcane plants from operating and from closed plantations were tested in 2001 and 2003 for SCYLV by TBIA. Open squares (□): no SCYLV was found, dark squares (■): SCYLV was detected at least in one sample. The star (☆) shows when YLS symptom outbreak was noticed for the first time. The plantations are ordered according to the island in which they are/were situated, the HSPA breeding station sites are plotted separately. The numbers in the squares denote the names (in short form) of the plantations (see also Table 1). *Hawaii*: 1 Kapoho, 2 Kohala, 3 Puna, 4 Hamakua, 5 Mauna Kea, 6 Kau. *Maui*: 1 Kaeleku, 2 Olowalu. 3 Lahaina, 4 HC & S (Puunene). *Oahu*: 1 Waianae, 2 Waimanalo, 3 Honolulu, 4 Laie, 5 Kahuku, 6 Oahu (Waipahu), 7 Waialua. *Kauai*: 1 Kilauea, 2 McBryde, 3 Anahola, 4 Kekaha, 5 Lihue, 6 G & R (Makawele). *HSPA breeding station*: 1 Lyon Arboretum, 2 Waianae quarantine, 3 Maunawili Pali Rd., 4 Kaaawa quarantine, 5 windbreaks, 6 export to Peru, 7 Maunawili hill side

SCYLV-types in sugarcane cultivars from Hawaii and clone R570 from Réunion

Four types of SCYLV had been identified so far (Moonan and Mirkov 2002; Abu Ahmad et al. 2006) and one plant had been analysed from the Hawaii collection, namely R570, which is a cultivar from Réunion. This plant contained the BRA-PER strain of SCYLV, not the REU-strain, which is typical for Réunion and which was found in R570 on Reúnion, Guadeloupe and Mauritius (Table 3). SCYLV of H87-4094 and H73-6110 was sequenced to analyse SCYLV of a Hawaiian cultivar grown in Hawaii. The sequences indicated that these two Hawaiian cultivars contain the PER type of SCYLV (data not shown).

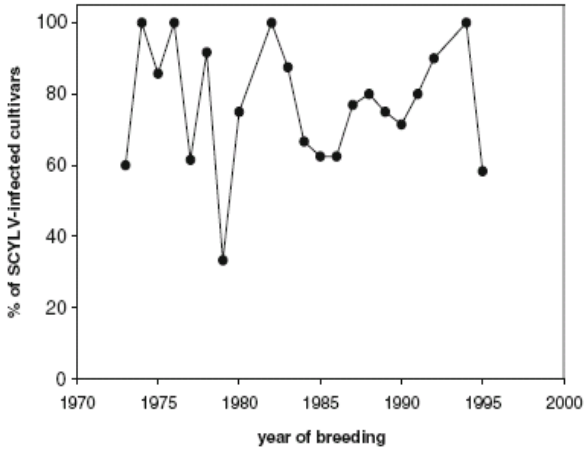

Fig. 6 Percent of SCYLV-infected Hawaiian cultivars from crossings in the years 1972–1986, tested in 2000

Discussion

The following picture emerged from this study: The HSPA breeding station became infested by SCYLV between 1960 and 1970 at a time when the plantations were still free of SCYLV. No hint as to which import(s) may have carried SCYLV into the breeding station was obtained. The aphid vector *Melanaphis sacchari*, which is present throughout Hawaii, will have disseminated the virus among the plants in the breeding station, infecting the cultivar collection and the newly-bred clones. Although the speed of infection propagation from plant to plant is only a few metres per year (Lehrer et al. 2007, Fig. 4), it is sufficiently fast to infect susceptible varieties within a few years. Four years was enough to infect 80% of the newly

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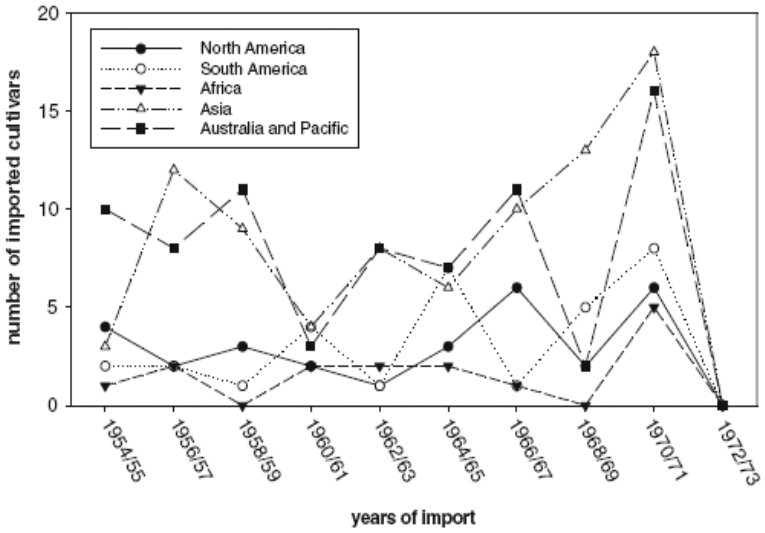

Fig. 7 Number of imported cultivars to the Hawaiian breeding station in the years 1954–1972 and their provenience

developed crossings at the Maunawili breeding station. Similarly a screening of clones at the breeding station Canal Point, Florida, had revealed that the SCYLV incidence increased during the CP sugarcane cultivar development program from 30% to 55% within 3 years (Comstock and Miller 2003). As a result of the many clones which were imported from around the world in the 1960s, several infected clones may have arrived in the breeding station, thus facilitating the uniform infestation of the entire station within a few years. The infestation may then have been carried over to the plantations by the field trials of new cultivars. The first reports of YL outbreak date to the late 1980s, also indicating that the plantations had not been fully SCYLV-infested in the years before.

The time range (1960–1970) where SCYLV is suspected to have arrived and spread in Hawaii is corroborated by a few other facts: the infected windbreak plants in the papaya plantations, the BRA-PER strain infection of the Hawaiian cultivars, which were exported to Peru in 1982, and finally the BRA-PER-strain of the variety R570 in Hawaii. That cultivar was imported from Réunion in 1981 and contains the BRA-PER strain (Abu Ahmad et al. 2006), although the same cultivar in Réunion harbors the REU strain and, in Mauritius, both REU and BRA-PER. Thus, Réunion was apparently SCYLV-free at 1981 when this cultivar was imported to Hawaii. Recent sequence analysis of SCYLV from Hawaiian sugarcane plants indicated that the strains group together with the PER-strain and are distinctly different from the South American BRA-strain, which is evidence that the Hawaiian cultivars grown now in Peru contained already SCYLV, when they were exported to Peru (ElSayed, pers. communication).

Table 3 SCYLV-strain in Hawaiian cultivars and in cultivar R570 from different sugarcane regions

Cultivar	Origin of tested material	Date of introduction	SCYLV-type
H87-4094	Hawaii	bred 1987	PER
H73-6110	Hawaii	bred 1973	PER
H50-7209	Peru	introduced 1982[b]	BRA-PER[a]
H32-8560	Peru	introduced 1982[b]	BRA-PER[a]
R570	Hawaii	introduced 1981	BRA-PER[a]
R570	Réunion	–	REU[a]
R570	Guadeloupe	introduced early 1980[c]	REU[a]
R570	Brazil	?	BRA-PER and REU[a]
R570	Florida	introduced before 1999[d]	BRA-PER
R570	Mauritius	introduced 1975	BRA-PER and REU[a]

[a] from Abu Ahmad et al. 2006
[b] from Alegria et al. 2000
[c] pers. communication J.-H. Daugrois
[d] pers. communication J. Comstock
[e] pers. communication S. Saumtally and K. Ramdoyal

There is no indication that the virus is eliminated by vegetative propagation of sugarcane. The infection is maintained through ratooning (Rassaby et al. 2004) and plants of several tested cultivars, for example cv. H87-4094, have kept the SCYLV-infection for at least 12–16 generations of successive seed piece plantings (Fig. 2). Therefore volunteer plants from closed plantations, which were found to be virus-free, were most likely already virus-free when the plantation was operating and had not lost a previous SCYLV-infection. Although the number of collected clones from the plantations and estates, which were closed in the 1940s and 1950s, was small so that by chance a resistant clone might have been among them, the large numbers of SCYLV-free clones of susceptible cultivars from Kahuku and from Kohala, both closed in 1970s, affirm that before 1970 the plantations were SCYLV-free.

An infection of plants after the time when the plantation was closed is very unlikely because a distance of only 100 m was already sufficient to prevent infestation from infected cane for at least 1 year (Lehrer et al. 2007) and a distance of several km was not bridged by viruliferous aphids for 30 years (Fig. 4). Most Hawaiian plantation estates were several km apart, except those at the Eastern Hawaii coast. In these, however, YL was already detected in the early 1990s (Schenck 1990), thus they were obviously already infected at that time. Therefore SCYLV-positive volunteer plants detected in fields which were given up in the late 1980s, indicate that the fields were indeed already infested at the time of closure.

The disease was not recognized for at least 30 years till it became obvious in the 1990s, it stayed unnoticed until it had spread through the plantations throughout the world. So far no suggestion existed as to when SCYLV infested the sugarcane plantations. The study presented here, using the fact that many sugarcane plantations and estates in Hawaii were closed in the past 50 years, gives a first hint that SCYLV entered the Hawaiian sugar industry in the 1960s through the breeding station and from there, in the 1980s, into the plantations. The SCYLV-infestation of Hawaii seemed to precede SCYLV-infestation of Réunion by more than 10 years. Also the Florida breeding station (Canal Point) may have been at least partly SCYLV-free in the 1970s, because cultivars, which were developed in and before that time and exported to Morocco, Spain or Thailand, were virus-free (Table 1). Samples from a Louisiana cultivar had thylakoid structures which were typical for SCYLV-infected plants (Robinson-Beers and Evert 1991; Yan et al. 2009), indicating that the virus was already present at the Louisiana breeding station at Houma in the 1980s. Thus it appears that all these important breeding stations acquired SCYLV in the 1970s or 1980s. It would also explain why sugarcane fields which had been isolated from cultivar exchange over the past 30 years (Uzbekistan, Syria) were SCYLV-free (Fig. 1); and why some old cultivars which had been exported 30 years ago to different places in the world were SCYLV-free (Table 1), unless they were infected later by more recent cultivar introductions.

Acknowledgements The study was supported by a grant of Deutsche Forschungsgemeinschaft. The gift of SCYLV-antibody by Dr. Lockhart (University of Minnesota) is gratefully acknowledged. We are also thankful to Drs. KK Wu, B. Osgood and S. Schenck (HARC, Aiea, USA) for their advice and help. Many researchers helped to obtain samples or tissue prints from sugarcane plants: J. Comstock, USDA, Florida, J. Hoy and L. Grelen, Louisiana, J.-H. Daugrois, CIRAD, Guadeloupe, R. Cu and F. dela Cueva, Philsurin, G.P. Rao, India, M. Casagrande, Brazil, J. Enahari, Morocco, T. van Antwerpen, South Africa, G. Khodjajeva, Uzbekistan, S. Ziad, Syria, B. Fontaniella, Spain.

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6. Molecular characterization of Hawaiian Sugarcane yellow leaf virus (SCYLV) genotypes and their phylogenetic relationship to SCYLV-strains from other sugarcane-growing countries

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Keywords: Luteoviridae, phylogenetic analysis, resistant and susceptible cultivar, RNA virus, Sugarcane yellow leaf virus (SCYLV), Yellow leaf

Submitted to *European Journal of Plant Pathology*

Abstract: *Sugarcane yellow leaf virus* (SCYLV) is the causal agent of the sugarcane disease Yellow leaf (YL), which was first reported in Hawaii. The presence of SCYLV was detected by tissue blot immunoassay and the Hawaiian sugarcane cultivars fell into susceptible cultivars (with SCYLV) and resistant cultivars (without SCYLV). RT-PCR showed recently that also the resistant cultivars contain the virus, however with a 100-fold lower virus titer than in the susceptible cultivars. SCYLV is present as whole genome (6kb) and as two subgenomic sequences of 2.4 and 1.0 kb. Virus preparations from three Hawaiian cultivars (two susceptible and one resistant) were fully sequenced and the sequences were aligned to published full and partial sequences. The phylograms corroborate previous findings that the so-called YLS-segment coding for the coat protein shows the least genetic diversity, whereas the other sequence fragments A-D, representing the ORFs 0-5, expressed a twofold higher diversity. The Hawaiian SCYLV-strains clustered together next to the Peru strain, apart from the BRA-strains and well apart from the REU-strains. We propose that the Hawaiian SCYLV should be considered as an independent group together with the Peru strain as HAW-PER. The sequences from the two susceptible cultivars had a deletion of 48 to 54 nt in ORF1, which codes for the gene silencing suppressor and a RNA-dependent RNA-polymerase. It is speculated that this deletion is important for the proliferation rate of the virus in the plant.

Introduction

The sugarcane disease Yellow leaf (YL) was first reported from plantations on two Hawaiian islands (Schenck, 1990). Few years later similar symptoms were observed in mainland US

(Comstock et al. 1994) and Brazil accompanied by dramatic yield losses (Vega et al. 1997). The symptoms are characterized by yellowing of leaf midribs followed by yellowing of the entire leaf blade and shortening of internodes of the green leaf top. Borth and Hu (1994) reported a dsRNA-virus in diseased plants. Later, a luteovirus (ss+RNA) could be unequivocally identified as causal agent of Yellow leaf (Vega et al. 1997) and it was named Sugarcane yellow leaf virus (SCYLV). Sequence analyses revealed that some regions of SCYLV genome are closely related to Barley yellow dwarf virus and others similar to the Potato leaf roll virus, which suggested that SCYLV may be a recombination product of a Polerovirus and an Enamovirus (Moonan et al. 2000, Smith et al. 2000). SCYLV-strains from different American countries were characterized by fingerprinting and partial sequence analysis and a Colombian strain was postulated as a founder strain of SCYLV (Moonan and Mirkov 2002). Later AbuAhmad et al. (2006, 2007) compared 60 SCYLV-preparations from almost all sugarcane-growing countries (including Colombia) by diagnostic PCR-reactions or by partial sequencing. SCYLV from Hawaiian cultivars were, however, not among that study, although YLS and SCYLV were first detected in Hawaii and the effect of SCYLV-infection on plant performance was already thoroughly studied for Hawaiian cultivars. Yet some SCYLV-preparations had a relationship to Hawaii, for example a SCYLV preparation from cultivar R570 which was grown in the collection of the Hawaiian sugarcane breeding station, contained the BRA-strain and not the REU-strain, which exists in R570 grown in Réunion (AbuAhmad et al. 2007). Similarly, the Hawaiian cultivars (H32-8560 and H50-7209), which were exported to Peru in 1981, were found to be infected with the PER strain, which is closely related to but not identical with the BRA-strain, the most common strain in many South and North American cultivars (AbuAhmad et al. 2006). Therefore, it was reasonable to assume that the sugarcane plantations of the Hawaiian Islands are infected by BRA and/or PER strains of SCYLV, however, direct evidence for this assumption is lacking because sequences of SCYLV from Hawaiian cultivars planted in Hawaii are not available so far. To date eight complete sequences of SCYLV are available plus more than 30 partial sequences, none from Hawaii. The Hawaiian cultivars were classified according to the presence of SCYLV into susceptible and resistant cultivars (Schenck and Lehrer 2000), based on the observation that all plants of susceptible cultivars contained SCYLV when tested by tissue blot immunoassay (TBIA), whereas plants from resistant cultivars appeared virus-free. The strength of YL-symptom expression was correlated (though not strictly) to the presence of SCYLV (Lehrer and Komor 2008). Recent data obtained by PCR indicated that the resistant Hawaiian cultivars also contained SCYLV although at very low titer (Zhu et al. 2010). The objective of this study was to sequence SCYLV from susceptible and resistant Hawaiian cultivars and to determine their phylogenetic relationship to SCYLV to already reported SCYLV clusters. In addition, although

the so-called YLS-segment of the SCYLV-sequence is considered as a valid diagnostic sequence for all SCYLV-strains (Comstock et al. 1998, Abu Ahmad et al. 2006, 2007), a reliable and accurate quantification of SCYLV in susceptible and resistant cultivars by RT-PCR or real-time PCR (Zhu et al. 2010) requires the accurate knowledge of SCYLV-sequences.

Material and Methods

Plant material and aphids

Cultivars H73-6110, H87-4319, H78-4153, H65-7052, H78-7750, and H87-4094 were obtained from the Hawaii Agriculture Research Center, Aiea, Hawaii, USA. A virus-free line of the cultivar H87-4094 was produced by meristem tip tissue culture and was provided by Dr. A. Lehrer, Honolulu. In addition, cultivars C1051-73, JA-605 and CP52-43, were obtained from Cuba through Medina Borges, Habana. The plants were grown in the greenhouse at Bayreuth University at 24°C with a 12-h photoperiod and propagated 1-2 times per year from cuttings.

Aphids *Melanaphis sacchari* were collected from sugarcane at Hawaiian Agriculture Research Center, Aiea, Hawaii, USA

Isolation of RNA, RT-PCR and northern blot for detection of SCYLV

RNA was extracted and purified from the top visible dewlap leaf as previously described (Comstock et al. 1998, Sambrook and Russell 2001, Lehrer et al. 2010).

RNA was extracted from aphids by the same protocol as described above with RNA extraction buffer (4M guanidine thiocyanate (Sigma-Aldrich, Chemie GmbH, Munich, Germany), 25 mM sodium citrate, pH 7.5% Sarkosyl (Sigma-Aldrich) and 2M sodium acetate pH 4.0).

RT-PCR was used to test the presence of SCYLV in the leaf samples of 9 Sugarcane cultivars and one aphid sample using diagnostic primers YLS111 and YLS462 (Comstock *et al.*, 1998). The RNA was reverse transcribed using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas GmbH, Leon –Rot Germany), primed with 50 pmol of YLS462 by following the manufacturer's protocol in a PCR machine (PTC 100 Peltier Thermal Cycler, MJ Research, Global Medical Instrumentation, Inc, Ramsey, Minnesota, USA.). The RT- PCR reaction was performed in 25 µl containing 1 µl cDNA, 2.5 µl of 10x PCR buffer containing 15mM $MgCl_2$, 0.5 µl of 10mM dNTP mix, 10 pmol each of forward and reverse primers (YLS111 and YLS462), 1 unit of a polymerase with proofreading activity (*Pfu*): *Taq* polymerase (5:1) (Stratagene, Waldbronn, Germany), and sterile milliQ water added up to the final volume of 25 µl. This PCR programme was performed with initial denaturation at 94°C for 4 min, 10 cycles of

94°C for 30 sec, 62°C for 2 min, 72°C for 1.5 min, and 30 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 1.5 min with a final 72°C extension for 7 min. The primer pairs located in the different ORFs of SCYLV genome are listed in Table 1 and Fig. 1.

Northern blots were prepared according Sambrook and Russell (2001) with 10 µg of intact RNA isolated from sugarcane leaves.

Genome fragment amplification

Genome fragments A-D, YL0, YL1, YL5 and YLS were amplified from reverse-transcribed RNA preparations as described above. The partial ORFs 0 and 1 (fragment A) was amplified from three cultivars (H73-6110, H87-4319 and H87-4094). The PCR program for the amplification of partial ORFs 0 and 1 with the primers ORF1START and 160R.640R was 94°C for 5 min, 10 cycles of 94°C for 30 sec, 62°C for 2 min, 72°C for 4 min, and 30 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 4 min with a final 72°C extension for 15 min. PCR program performed with the primers oFM323 and oFM359 was 94°C for 5 min, 10 cycles of 94°C for 30 sec, 58°C for 2 min, 72°C for 4 min, and 30 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 4 min with a final 72°C extension for 15 min. Partial sequence of ORF2, ORF5 and complete sequence of ORF3 and ORF4 (Fragment C) were amplified with three cultivars (H73-6110, H87-4319 and H87-4094). The RT-PCR program performed with primers B FOR and B REV was 94°C for 5 min, 10 cycles of 94°C for 30 sec, 62°C for 2 min, 72°C for 4 min, and 30 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 4 min with a final 72°C extension for 15 min.

The partial sequence of ORF5 (fragment D) was amplified from RNA isolated from the cultivars H73-6110, H87-4319 and H87-4094 with 104R.623R and 3´PRIME2 primer pair. The RT-PCR program was 94°C for 5 min, 10 cycles of 94°C for 30 sec, 62°C for 2 min, 72°C for 4 min, and 30 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 4 min with a final 72°C extension for 15 min.

PCR reaction for amplification the gap in ORF1 in SCYLV genome (fragment YL1) was performed with the YL1FOR and YL1REV primer pair to cover the non-sequenced region. The PCR programme was the same as for primers for YLS. The amplification of the region in ORF0 in SCYLV genome (fragment YL0) was performed with primer ORF0 FOR and ORF0 REV and the PCR programme was the same as mentioned above with primer YLS. Additionally, the fragment YL5 was amplified with primer ORF5 FOR and ORF5 REV and the PCR programme was the same as mentioned above with primer YLS.

The RT-PCR was performed with internal control, 25srRNA, as a reference gene to normalize gene expression level and to evaluate the integrity of cDNA. Furthermore, the primer sets were optimized using semi qPCR with different numbers of PCR-cycles.

A 15 µl aliquot of each amplified product was analysed by electrophoresis on 1% agarose gels stained with ethidium bromide.

Cloning and sequencing of RT-PCR products

Twenty-five amplicons of all viral fragments, derived from independent RT-PCR reactions were cloned in the pGEM®-T Easy Vector System (Promega, Mannheim, Germany) and were transformed into the competent *E. coli* DH5α strain. The recombinant DNA clones containing the inserts were purified using the Pure yield™ Plasmid Miniprep System (Promega, Mannheim, Germany). The selected clones were sequenced by primer walking using M13 sequencing primers and internal primers specific for each of the fragments A, B, C, and D in the DNA Analytics Core Facility at the University of Bayreuth. One clone per amplicon was sequenced and used for alignment and phylogenetic analyses.

Alignment of sequences and construction of phylogenetic trees

Multiple sequence alignments of nucleotide or deduced amino acid sequences were aligned using CLUSTAL W applying the Dayhoff PAM 250 matrix (Thompson et al. 1994) and were optimized manually. Phylogenetic reconstructions were performed using Geneious program, version 4.7.5 (www.geneious.com). Trees were constructed by the UPGMA method. Data sets were bootstrapped (1,000 replicates) to assess the confidence values of the phylogenetic trees, and bootstrap values < 50% were omitted. The resulting sequences were compared with the GenBank database (NCBI). The GenBank accession numbers of the sequences determined here and those used for phylogenetic analysis are listed in Table 2.

Results

Detection of SCYLV in Hawaiian cultivars using RT-PCR and northern blot analysis

RNA was isolated from ten cultivars (seven from Hawaii and three from Cuba) and used for cDNA synthesis and SCYLV detection. The diagnostic primers YLS111 and YLS462 were used in the PCR reactions (AbuAhmad et al. 2006). A virus-free line of cultivar H87-4094 was generated by meristem tip culture and used as negative control. As expected, two susceptible Hawaiian cultivars (H73-6110 and H87-4094) showed strong amplification products of SCYLV of the expected size, while the virus-free line H87-4094Vf showed no PCR product (Fig. 1). The

three resistant cultivars (H87-4319, H78-4153 and H78-7750) showed a quite different amplification patterns. While H78-4153 and H78-7750 generated a weak band corresponding to the correct size, H87-4319 expressed a relatively strong amplification product. The three Cuban cultivars (C1051-73, JA-605 and CP52-43) showed weak bands of SCYLV, similarly aphids feeding on cv. H87-4094. Using different numbers of RT-PCR cycles, the differences in virus titer between susceptible and resistant cultivars could be estimated to be at least 100-fold (data not shown).

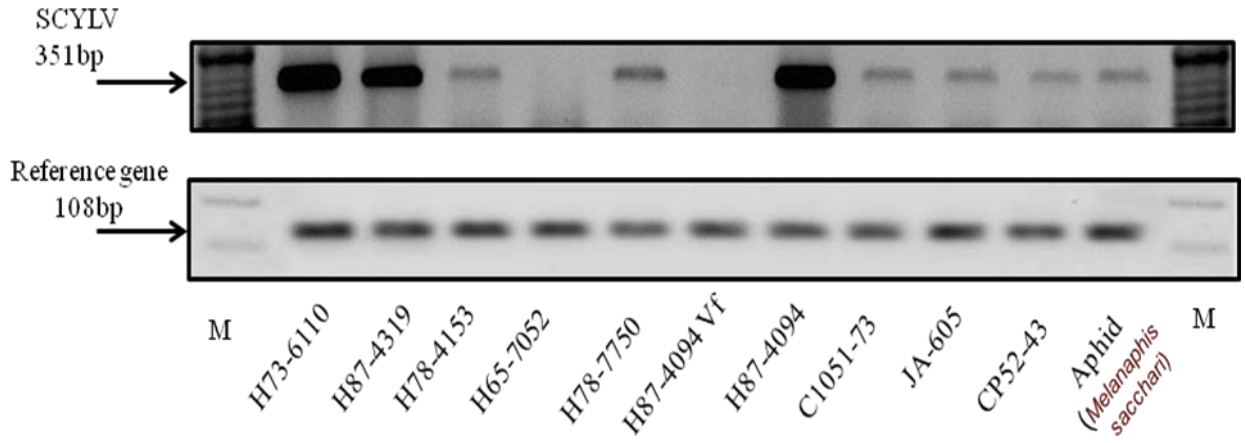


Fig. 1 RT-PCR for SCYLV in ten Hawaiian cultivars, three Cuban cultivars and viruliferous *Melanaphis sacchari*. RNA from leaves of cultivars and aphids was extracted, transcribed to cDNA and amplified with diagnostic primers (YLS111 and YLS 462) by RT-PCR. H73-6110 and H87-4094 are susceptible, H65-7052 intermediately susceptible and H78-4153, H87-4319 and H78-7750 resistant cultivars. The virus-free clone of H87-4094 is used as a negative control, in addition three Cuba cultivars, C1051-73, JA-605 and CP52-43 were tested. The PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide. (M: DNA molecular size marker). Loading control 25SrRNA, 108bp.

Northern blot analysis revealed an accumulation of SCYLV at high level in the lines H73-6110, H87-4094 and H87-4319 (Fig. 2). The cultivars H78-4153, H65-7052 and H78-7750 showed no signal indicating no virus or a low titre below the detection threshold. The genome of SCYLV apparently contained genomic RNA (gRNA) and two subgenomic RNAs (sgRNAs). The estimated molecular size of the gRNA was 6.0 kb, the estimated molecular sizes of the sgRNAs were 1.0 and 2.4 kb.

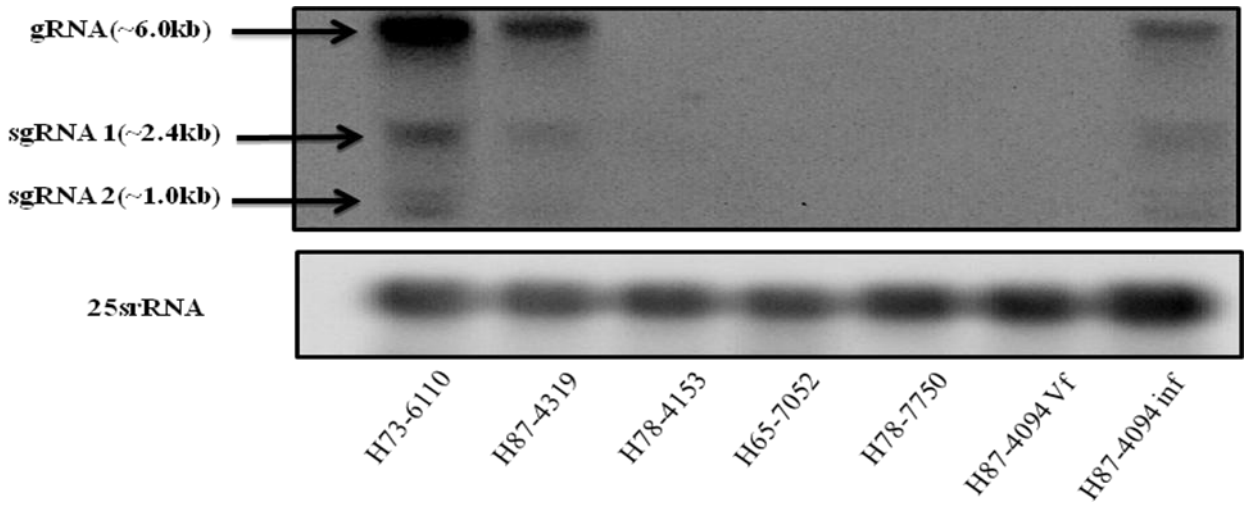


Fig. 2 Northern blot of SCYLV isolated from Hawaiian sugarcane. The RNA gel blot was probed with DIG-labeled SCYLV probe covering the YLS sequence part and detected with anti-digoxigenin-AP and CDP-*Star* ready-to-use and visualized with a chemilux CCD camera (Intas, Göttingen-Germany). The apparent size of the hybridization signals was deduced from RNA molecular size markers (not shown), loading control 25SrRNA visualized by probe hybridization.

Fragment amplification of SCYLV isolates and phylogenetic relationship to published SCYLV-isolates

SCYLV is a ss+-RNA virus with 5895-5899 nucleotides organized in six open reading frames (ORFs 0-5) (Fig. 3). Eight primer pairs for amplification of fragments YLS and A-D were designed (Table 1) for the six open reading frames. Complete sequences of SCYLV from 3 Hawaiian cultivars and several partial sequences (accession numbers: GU570004, GU570005, GU570006, GU570007, GU570008, GU570009, GU570010) were obtained.

The Hawaiian amplicons were used for phylogenetic analysis together with sequences from the GenBank data base (http://www.ncbi.nlm.nih.gov) (Table 2). Since the SCYLV-genome is a recombination product of two *Luteoviridae* viruses, the phylogenetic relationship of fragments A-D and YLS were separately constructed to visualize possible sequence segments, where the Hawaiian strains may have diverged from other strains.

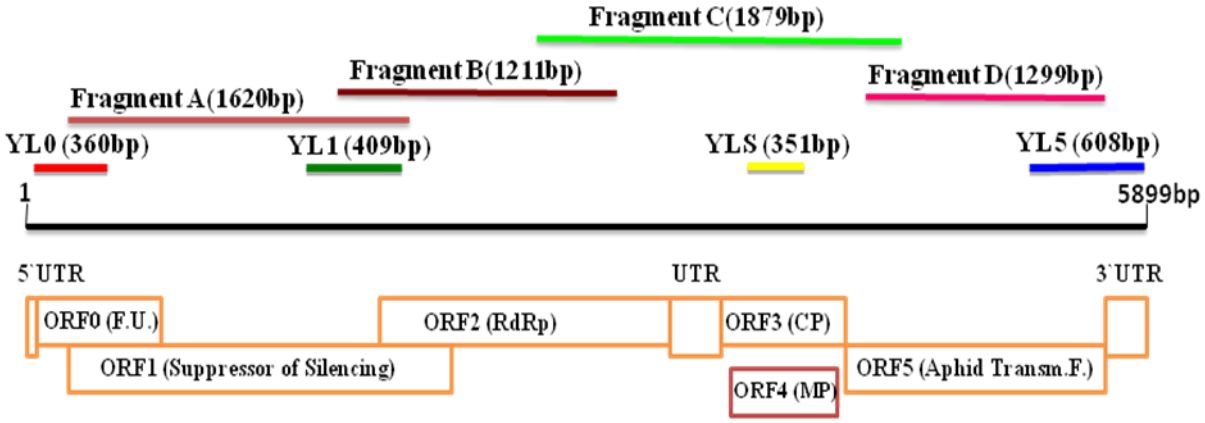


Fig. 3 SCYLV genome organization, functional open reading frames and positions of amplified SCYLV fragments. aphid transmission F. = putative aphid transmission factor, **CP** = capsid protein, **MP**=putative movement protein, **ORF**: open reading frame, **RdRp** = RNA-dependent RNA-polymerase, **UTR**: untranslated region.

Table 1. Primers which were used for sequence fragment amplification.

Primers name*	5´ - 3` sequence	Amp. Fragment and location in the genome
ORF1START	ATGGCCCCAACACTCCCGTTTACA	***A*** (partial ORF0 and 1)
160R.640R	GAATCAACTGCGAGACGATG	
oFM323	CAGACATTGCTGATTAC	***B*** (partial ORF2)
0FM359	GCTCTCCACAAAGCTATCT	
B FOR	GGATTGTGCGATCCGATTCG	***C*** (ORFs 3 and 4, partial ORFs 2 and 5)
B REV	CAGTTGCTCAATGCTCCACG	
104R.640R	ATATCTAGATGTGGGTCCGC	***D*** (partial ORF5)
3`PRIME2	ATATCTAGATGTGGGTCCGC	
ORF0FOR	TTTGGACCAAGCCTCTGACT	**YL0** (partial 5`UTR and ORF0)
ORF0REV	GGCAAGCCATAAAAGGACAG	
ORF5 FOR	GCCGACACTTTAAGACAGGC	**YL5** (3`UTR and partial ORF5)
ORF5 REV	TTAGCTTGGGCTTCCAAAGA	
YL1FOR	CGGCGCCTAATTTTGTGTAT	**YL1**(partial ORF1)
YL1REV	GAATCAACTGCGAGACGATG	
YLS111	TCTCACTTTCACGGTTGACG	**YLS** (partial ORFs 3 and 4)
YLS462	GTCTCCATTCCCTTTGTACAGC	

*Primers in italics were used according to Abu ahmed *et al.*, 2006.

Table 2. SCYLV sequences used in the phylogenetic analysis.

Isolate name*	Source of SCYLV	Country/location of origin	GenBank accession number
Aus1	VMC71-238	Australia	AJ491255
Aus2	VMC71-238	Australia	AJ491256
Beaz1	SP77-5181	Brazil	AJ491274, AJ491257
BRA1	SP83-5073	Brazil	AJ582772
BRA2	RB835054	Brazil	AJ582779
BRA-YL1	SP71-6163	Brazil	AM072750
CBB38192	B38192	India:Coimbatore	EF635935
CB671	CoC671	India:Coimbatore,	EU624499
CB86032	Co86010	India:Coimbatore	EU624497
CB99016	Co99016	India:Coimbatore	EU089687
CBAPHIDS	Aphids	India:Coimbatore	EU089688
CHN-YL1	CGT63-167	China	AM072751
COL4	SP71-6163	Colombia	AM072624
CP52-43	*CP43-62*	*Cuba*	*GU570009*
CP65-357	CP65-357	USA	AJ249447
CPam1	CP65-357	Australia:Queensland	AJ491271
CPaus1	CP65-357	Australia:Queensland	AJ491270
CUB1	JA64-11	Cuba	AJ621181
CUB2	C13-281	Cuba	AJ582770
CUB-YL1	C132-81	Cuba	AM083988
Haw1	H78-3606	USA:Hawaii	AJ491261,AJ491118,AJ491278
Haw2	H87-4094	USA:Hawaii	AJ491262,AJ491119,AJ491279
Haw3	H78-7750	USA:Hawaii	AJ491120
Haw73-6110	*H73-6110*	*Hawaii:USA*	*GU570008*
Haw73-6110b	*H73-6110*	*Hawaii:USA*	*GU570005, GU570004*
Haw87-4094	*H87-4094*	*Hawaii:USA*	*GU570006*
Haw87-4319	*H87-4319*	*Hawaii:USA*	*GU570007*
HawAphids	*Aphids*	*Hawaii:USA*	*GU570010*
Hy-IND-1	Co 62399	India	FJ430665
KER-IND-1	Co 7219	India	FJ430661
KER-IND-2	Co C671	India	FJ430662
Maur1	M1658-78	Mauritius	AJ491280, AJ491263, AJ491140
Maur2	M2350-79	Mauritius	AJ491264
MUS1	M99/48	Mauritius	AJ606085
MYS1	TC4	Malaysia	AJ606084
PER1	H32-8560	Peru	AM072627, AJ621179, AJ582767
PER-YL1a	H50-7209	Peru	AM072752
PER-YL1b	H50-7209	Peru	AM072753
PER-YL1b	H50-7209	Peru	AM072753
PHL1	VMC76-16	Philippines	AJ582761
REU1	R570	Re′union Island:LeGol	AJ621163
REU12	R569	Réunion Island (Vue-Belle)	AJ606087, AJ582765, AJ582791
REU13	M1371/79	Réunion Island (Vue-Belle)	AJ606088, AJ582769, AJ582783
REU15	R579	Réunion Island (St-Benoît)	AJ621165, AM072633
REU17	R576	Réunion Island (St-Benoît)	AJ606089
REU2	R577	Réunion Island (Le Gol)	AJ621166, AJ582785
REU22	R570	Réunion Island (St-Benoît)	AJ621168,AM072638
REU3	R577	Réunion Island (Le Gol)	AJ606090, AJ582773, AJ582786
REU31	AY7	Réunion Island (La Mare)	AJ621171,AJ582781,AJ582788, AM072644

Table2 continued

Isolate name*	Source of SCYLV	Country/location of origin	GenBank accession number
REU32	R579	Réunion Island (La Mare)	AJ606091
REU33	R490	Réunion Island (La Mare)	AJ621172, AM072645
REU35	R577	Réunion Island (Vue-Belle)	AJ582787, AM072647
REU37	R81-0834	Réunion Island (La Mare)	AJ582775, AM072648
REU39	AY7	Réunion Island (La Mare)	AJ621175, AM072650
REU40	SP71-6163	Réunion Island (La Mare)	AJ621177, AJ582782, AJ582784
REU42	SP71-6163	Réunion Island (La Mare)	AJ621159, AJ582762, AJ582792
REU48	S17	Réunion Island (La Mare)	AM072656
REU5	M1371/79	Réunion Island (Vue-Belle)	AJ582763
REU7	CP70-1133	Re′union Island:Vue-Belle	AJ621161
REU9	R575	Réunion Island (Vue-Belle)	AJ606092
Reun-1	R84-0408	Reunion	AJ491282, AJ491265
Reun-2	R85-1102	Reunion	AJ491283, AJ491266
REU-YL1a	R570	Re′union Island :La Mare	AM072754
REU-YL1b	R570	Re′union Island :La Mare	AM072755
REU-YL2	R490	Re′union Island :La Mare	AM072756
REU-YL3	SP71-6163	Re′union Island :La Mare	AM085306
SCYLV C3	CC85-964	Colombia: Cali	AF369928
ScYLV-A	CP65-357	USA:Florida	AF157029
ScYLV-B1	SP71-6163	Brazil:Sao-Paulo	AF369925
ScYLV-C1	SP71-6163	Colombia:Cali	AF369927
ScYLV-C3	CC85-964	Colombia:Cali	AF369928
ScYLV-C4	CC84-75	Colombia:Cali	AF369929
ScYLV-F	CP65-357	USA:Florida	AJ249447
ScYLV-G2	CP92-1654	Guatemala:Santa-Lucia	AF369924
ScYLV-IND	-	India:Gorakhpur	AY236971
ScYLV-L1	LHo83-153	USA:Baton Rouge	AF369923
ScYLV-N6	Q136	Argentina:Santa-Rosa	AF369926
Taiw1	ROC11	Taiwan	AJ491144, AJ491127
Taiw2	ROC12	Taiwan	AJ491269
TN-IND-1	Co 86010	India	FJ430663
TN-IND-2	Co 93009	India	FJ430664
TWN1	ROC6	Taiwan	AM072630
USA1	TCP87-3388	USA:Florida	AM072631
USA1a	CP81-1405	USA	AJ491114
USA1b	CP81-1405	USA	AJ491115
USA2	CP85-1491	USA:Florida	AJ621162
USA2a	CP88-1409	USA	AJ491276, AJ491116
USA2b	CP88-1409	USA	AJ491117, AJ491277

*Isolates in italics were amplified and sequenced in this study and other data from GenBank.

The nucleotide sequences of the YLS-region were obtained from 44 SCYLV isolates. The Hawaiian sequences were assembled in cluster YLS1 (Fig. 4a). The resistant cultivar (Haw87-4319) showed 100% sequence identity to YLS from Brazil, Florida, China and Réunion. the YLS-sequence from the two susceptible Hawaiian cultivars (Haw73-6110 and Haw87-4094) exhibited close sequence similarity to those from Australian and Peru. Only SCYLV from Indian cultivars (and one Colombian) were clustered separately from all other YLS-sequences (cluster

YLS2 in Fig. 4a). The deduced amino acid sequences of the capsid protein (CP) obtained from all the isolates expressed almost identical amino acid sequences (97-100%, data not shown).

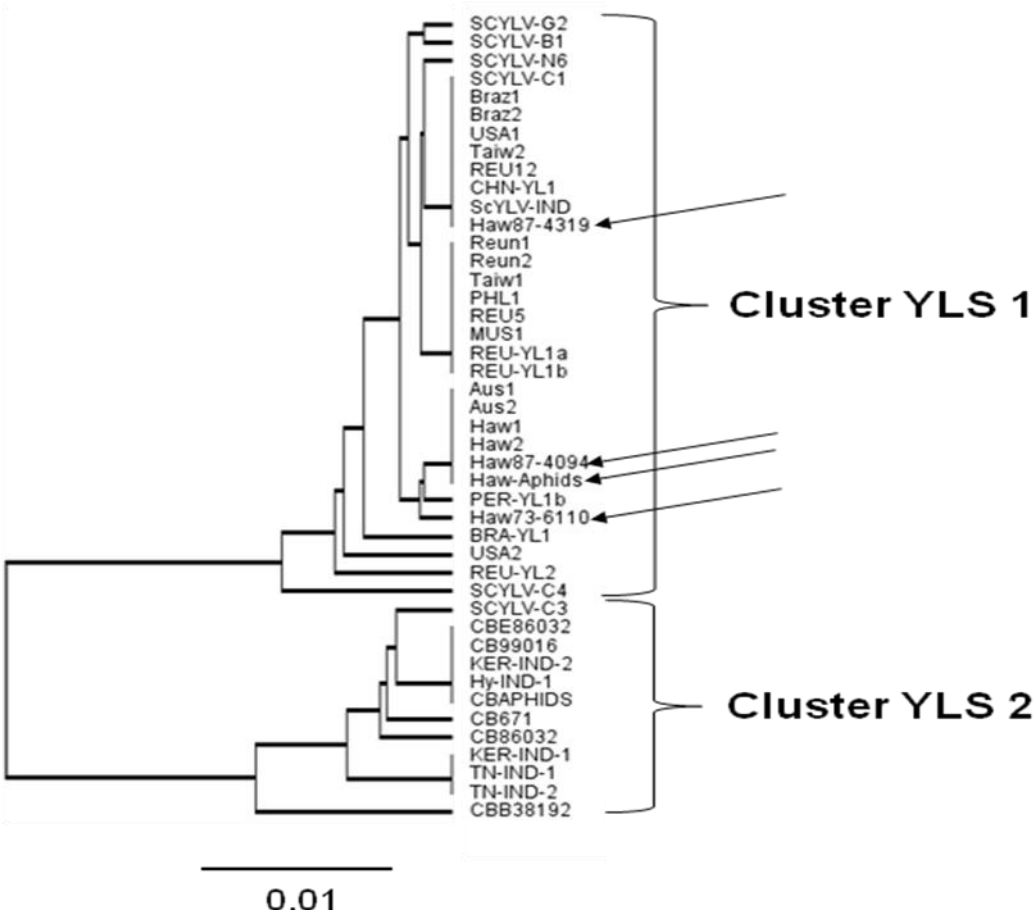
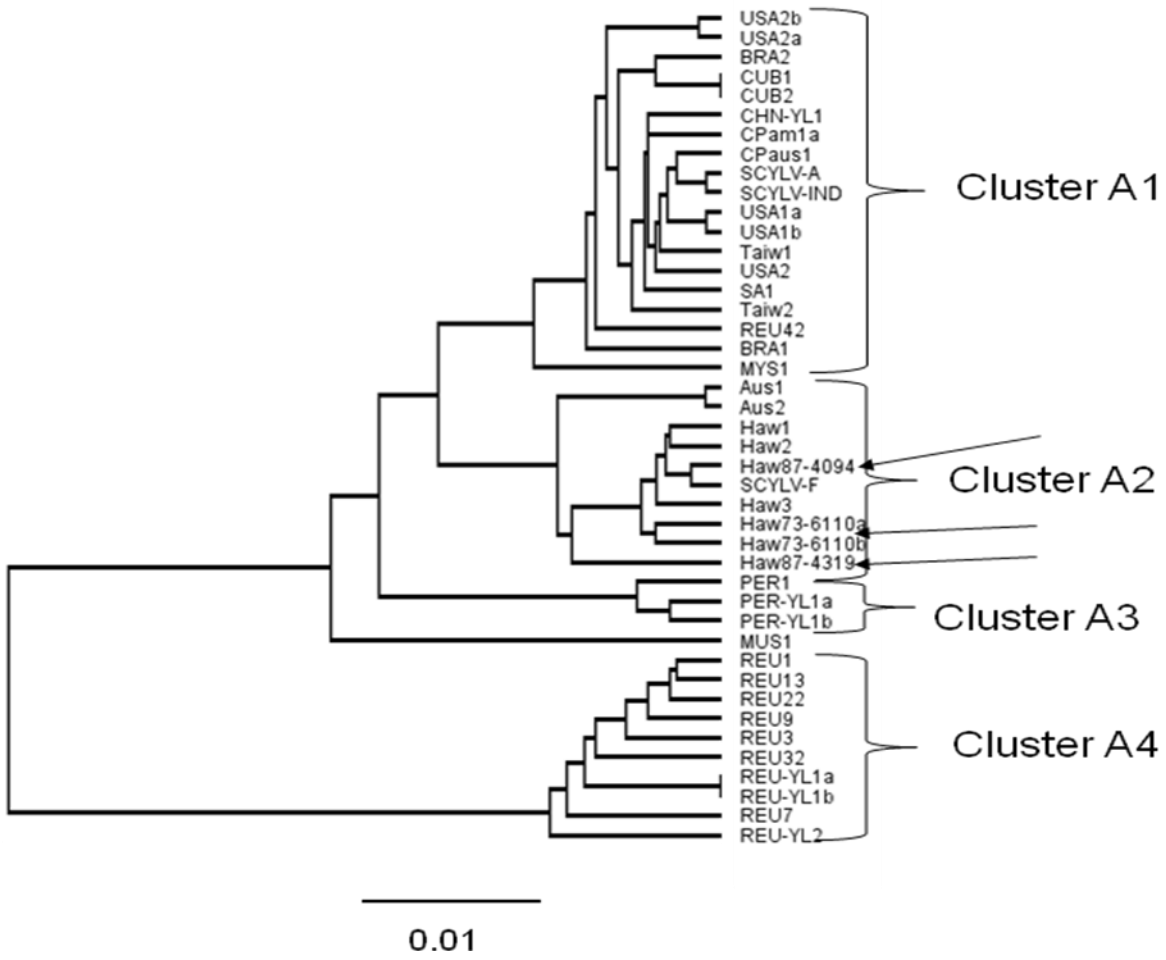


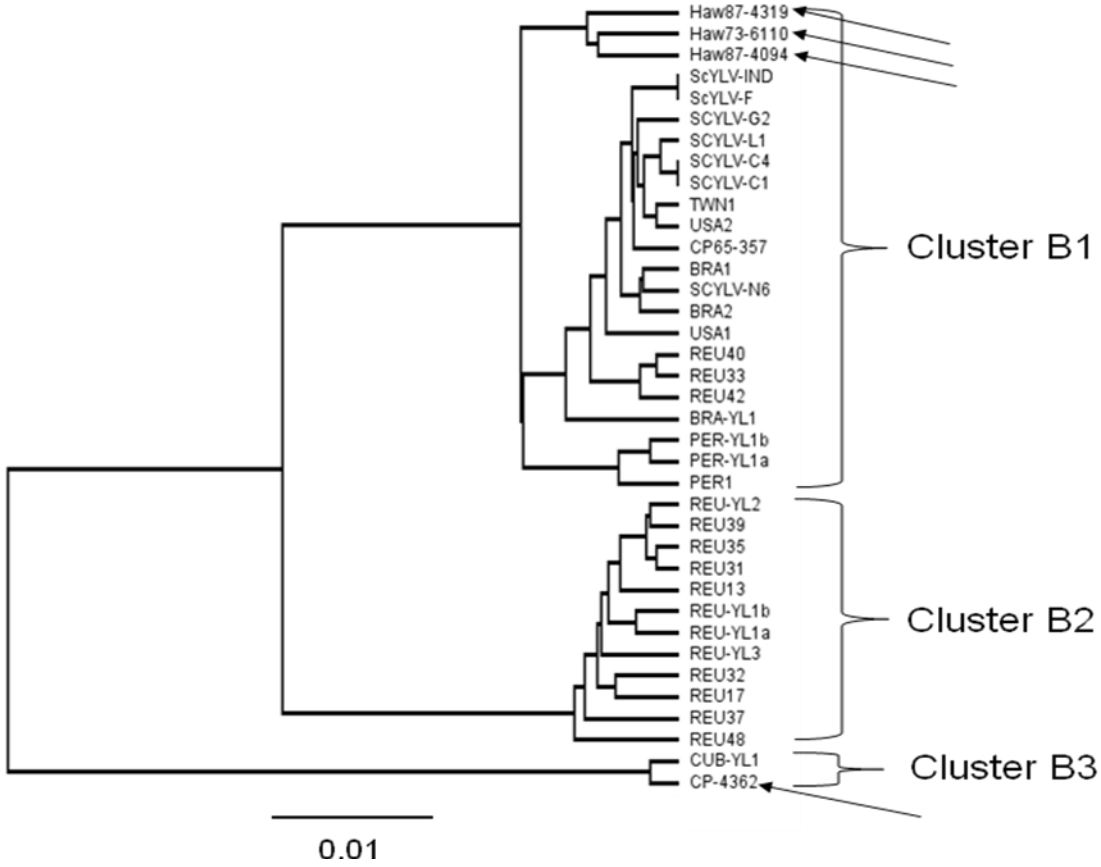
Fig. 4a Phylogenetic trees based on nucleotide sequence alignments of SCYLV isolates. The trees were constructed with Geneious program and UPGMA method. Numbers above the lines indicate the bootstrap scores out of 1,000 replicates. **a)** Phylogram of fragment YLS (351 nt),

Forty-three virus isolates were used in phylogenetic analysis of fragment A (comprising partial ORFs 0 and 1). The sequences were distributed into three major groups (Fig.4b). Cluster A1 contained 19 SCYLV sequence isolates from many origins (USA, Brazil, China, Australia etc.), the Hawaiian isolates were together in cluster A2 and the Peru isolates (A3) appeared similarly close to A1 (BRA) and Hawaiian isolates (A2). The sequence identity among Hawaiian isolates ranges between (91.2% and 98.9%). The majority of Réunion virus strains were clustered into group A3. (MUS1 seemed to be unique not fitting in any of the above groups.)



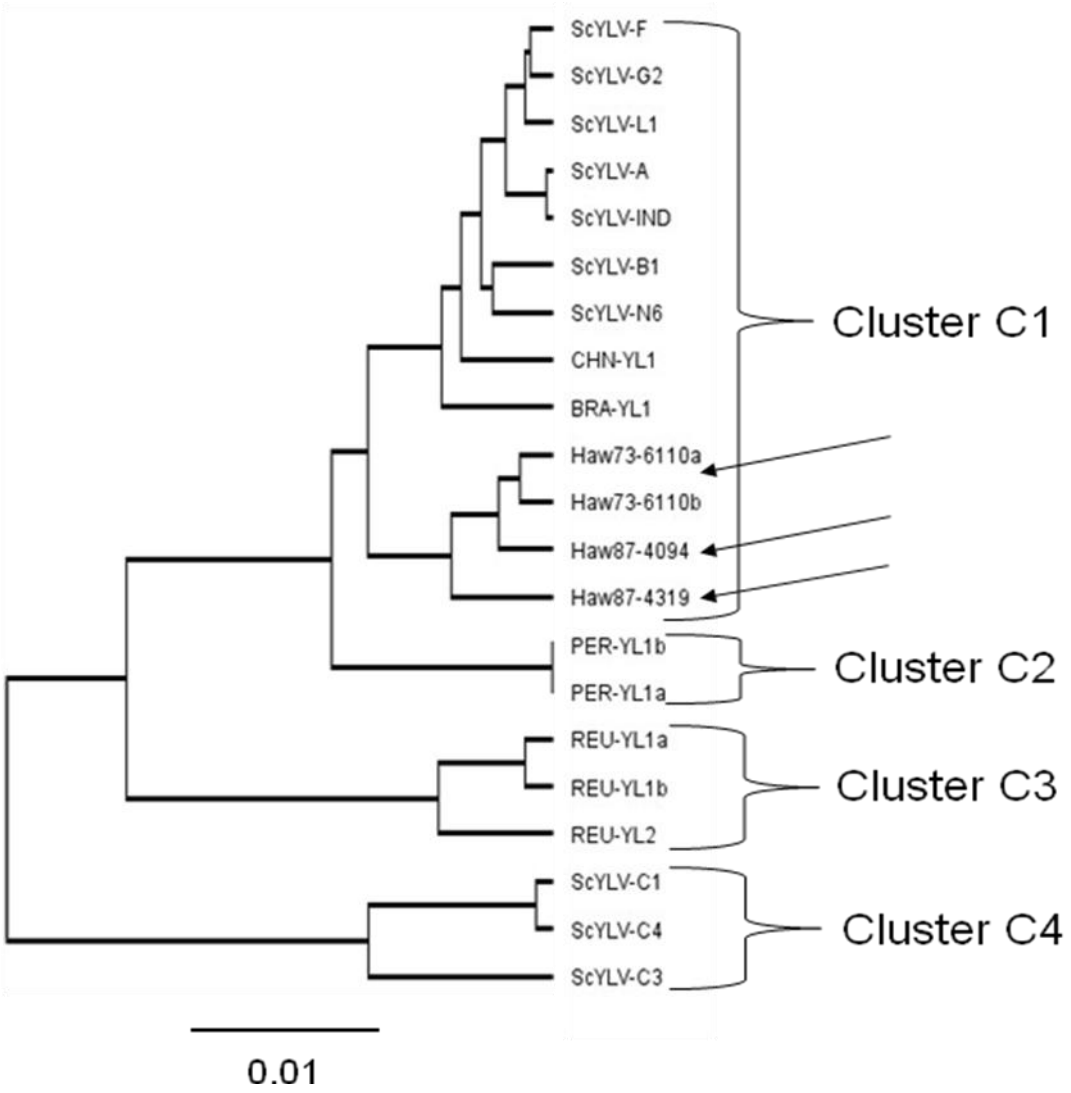
4b) Phylogram of fragment A (1620 nt),

Thirty-seven sequences of fragment B (partial ORF2) were classified into three clusters (Fig.4c), the Hawaiian virus isolates (Haw73-6110, Haw87-4319 and Haw87-4094) clustered in B1 as unique subgroup with a sequence identity between 89.5% and 90.3%. The Réunion isolates were grouped together in cluster B2, cluster B3 contained two Cuban virus isolates (CUB-YL1 and CP52-43) with 98.7% identity.



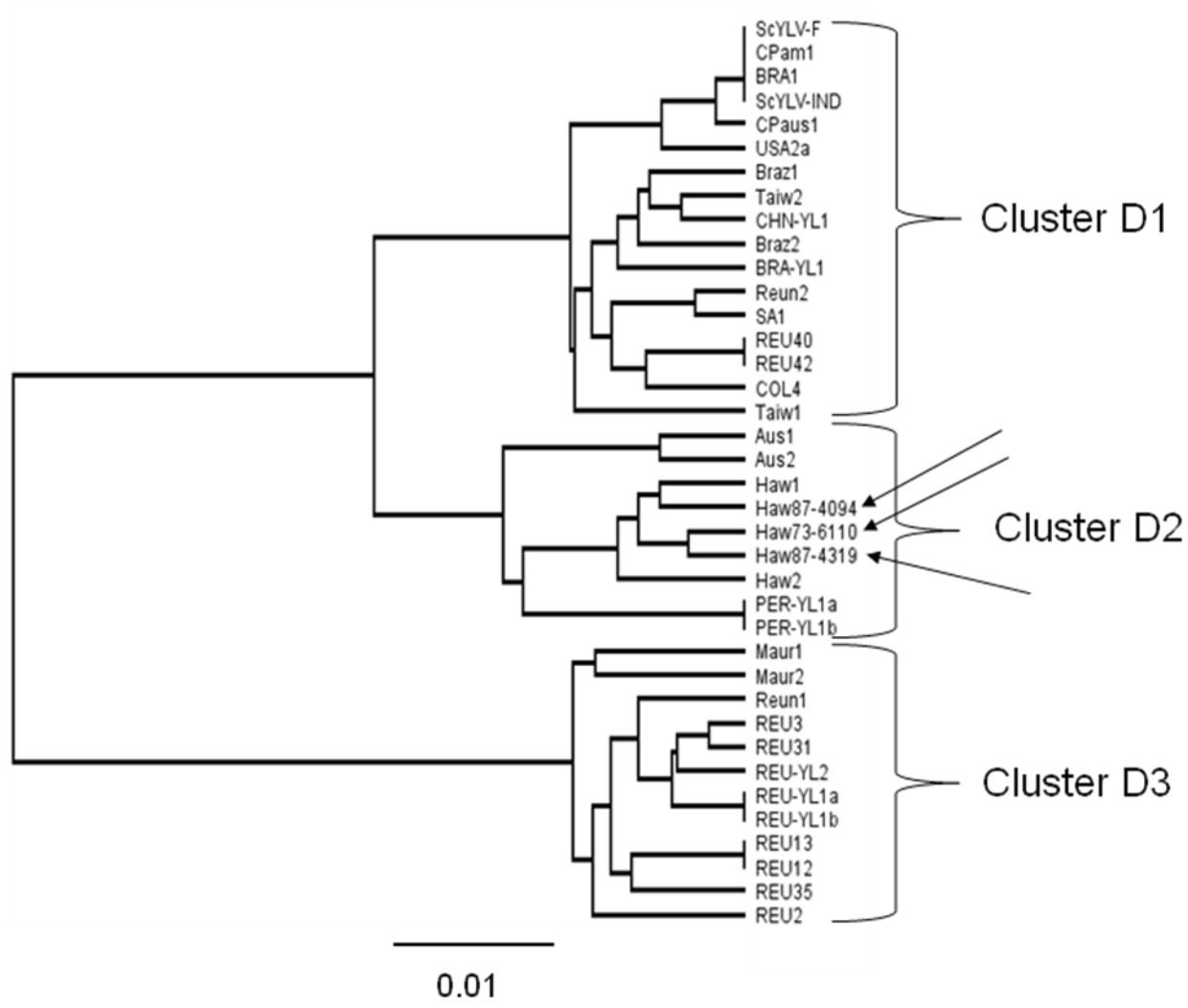
4c) Phylogram of fragment B (1211 nt),

The sequences of 21 isolates of fragment C, which covers ORFs 3 and 4 and parts of ORFs 2 and 5, were assembled into 4 clusters (Fig.4d). Nine SCYLV-isolates from different geographical origins (Brazil, USA etc.) were grouped in cluster C1 together with the Hawaiian isolates, cluster C2 contained the PER-isolates, REU-isolates were assembled into group C3 and cluster C4 included only Colombia isolates.



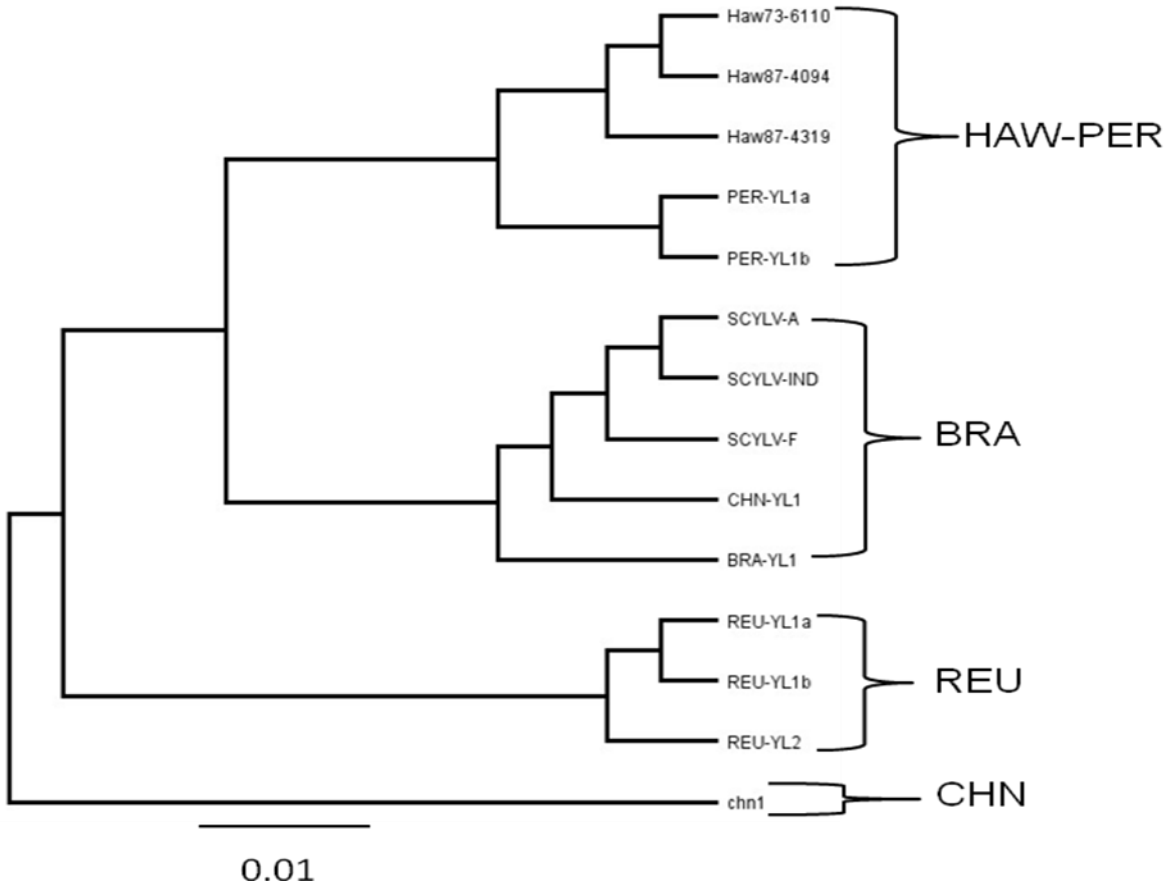
4d) Phylogram of fragment C (1879 nt),

Thirty-eight SCYLV isolates of fragment D which is related to the putative aphid transmission factor, were grouped into three clusters (Fig. 4e). Cluster D1 contained subgroups from various origins of SCYLV isolates (e.g. Florida, Brazil and India in one subgroup, isolates REU40 and REU42 in another subgroup). Hawaiian isolates were assembled in cluster D2 as unique subgroup, the majority of Réunion virus isolates was clustered in D3 together with two isolates from Mauritius.



4e) Phylogram of fragment D (1299 nt),

The phylogenetic analysis of the SCYLV partial sequences constantly yielded 3 clusters: (1) a big group comprising strains from Brazil, USA, China, India and several other countries (tentatively group BRA according AbuAhmad et al. 2006), (2) a group exclusively for most strains from Réunion (group REU) and (3) a group with the Hawaiian and the Peru strains. For this group we propose the name HAW-PER. In fragment C a Colombian cluster, in fragment B a Cuban cluster showed up, separated from the other strains. Phylogenetic analysis of 14 complete sequences of SCYLV genome also exhibited three groups: Group HAW-PER included two subgroups with the Hawaiian and Peru isolates with bootstrap value 89%., group 2 (BRA) formed by isolates from various origins (Brazil, China, India and USA) and group 3 with the REU strains. Thus the whole genome confirms the results already obtained from the alignment of partial sequences which are available in much larger numbers.



4f) Phylogram of SCYLV complete genomes

Deletion/insertion in ORF1

The nucleotide sequences from susceptible cultivars H78-6110 and H87-4094 and the resistant cultivar H87-4319 showed a lack of 48 to 54 nucleotides in the susceptible cultivars (Fig. 5a). A 51 nt deletion was detected in fragments A and B of cultivar H87-4094 corresponding to nucleotides 1686 to 1736 of SCYLV (NCBI accession NC_000874, Moonan et al. 2000). In contrast, a 48 nt deletion was detected in two independent A fragments obtained from cultivar H73-6110 corresponding to nucleotides 1686 to 1733 of SCYLV and a 54 nt deletion corresponding to nucleotides 1681 to 1734 in fragment B. Since these deletions were detected in independent amplification products of these two sugarcane cultivars, they did most likely not result from amplification and cloning artifacts or from sequencing errors. In addition, the detection of a 48 nt and a 54 nt deletion in amplification products from cultivar H73-6110 might indicate the presence of two SCYLV genotypes in this plant line. RT-PCR with primers flanking this particular region yielded amplification products of the expected size; 409 bp from the resistant cultivar H87-4319 and about 359 bp from the susceptible cultivars H73-6110 and H87-4094 (Fig. 5b). When a few other cultivars were tested with the same primer combinations, H78-7750 also showed the deletion, whereas two cultivars obtained from Cuba, JA-605 and CP52-43

contained the 50nt stretch (Fig. 5b). The deletion in SCYLV from susceptible cultivars lies in the ORF1 for a "multifunctional protein" which is thought to be involved in suppression of gene silencing, and at a cleavage point of RNA-dependent RNA polymerase (RdRp, ORF1 to ORF2). The amino acid sequences of RNA-dependent RNA polymerase (RdRp) from fully sequenced SCYLV-strains showed lower sequence identities in the first half and high identity in the second half of the protein (Fig 6). The 16-18 aa gap of the two isolates H73-6110 and H87-4094 lies just in between of these two halves.

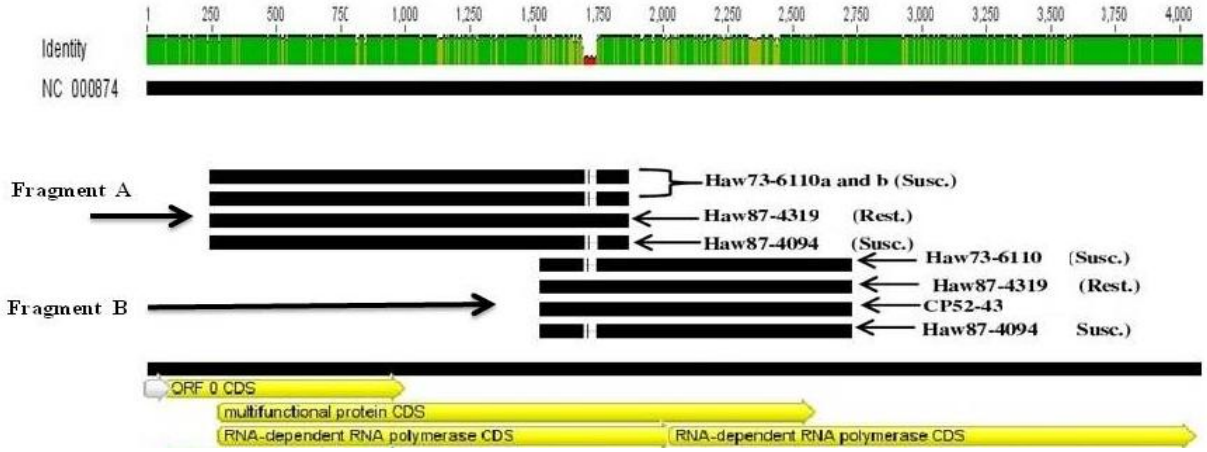


Fig. 5a Sequence gap in SCYLV from susceptible cultivars a: Location of sequence gap in SCYLV from susceptible cultivars versus SCYLV from resistant cultivar and ORFs for coded proteins. The gap was in overlap of fragments A and B, the deletions were in susceptible cultivars only.

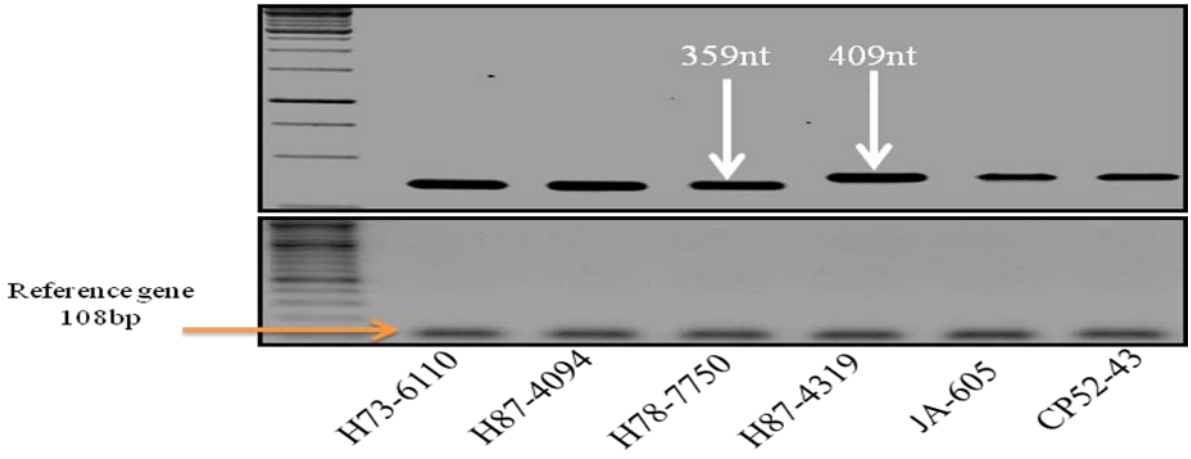


Fig.5b: RT-PCR of the sequence segment in ORF1 which contains the deletion in some cultivars. Primers YL1FOR and YL1REV were designed to amplify the sequence nt1211-1620 from RNA-preparations of sugarcane leaves as templates. Cultivars H73-6110 and H87-4094 are susceptible, cvs. H87-4319 and H78-7750 are resistant. Cvs. H78-7750, JA-605 and CP52-43 were infected with viruliferous *Melanaphis sacchari*. M: 1kb and 50bp DNA molecular size markers (Fermentas, St. Leon Rot, Germany). The lower panel shows the transcription of ribosomal RNA in the same preparation to demonstrate the activity of cDNA amplification (108bp from25srRNA). The PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide.

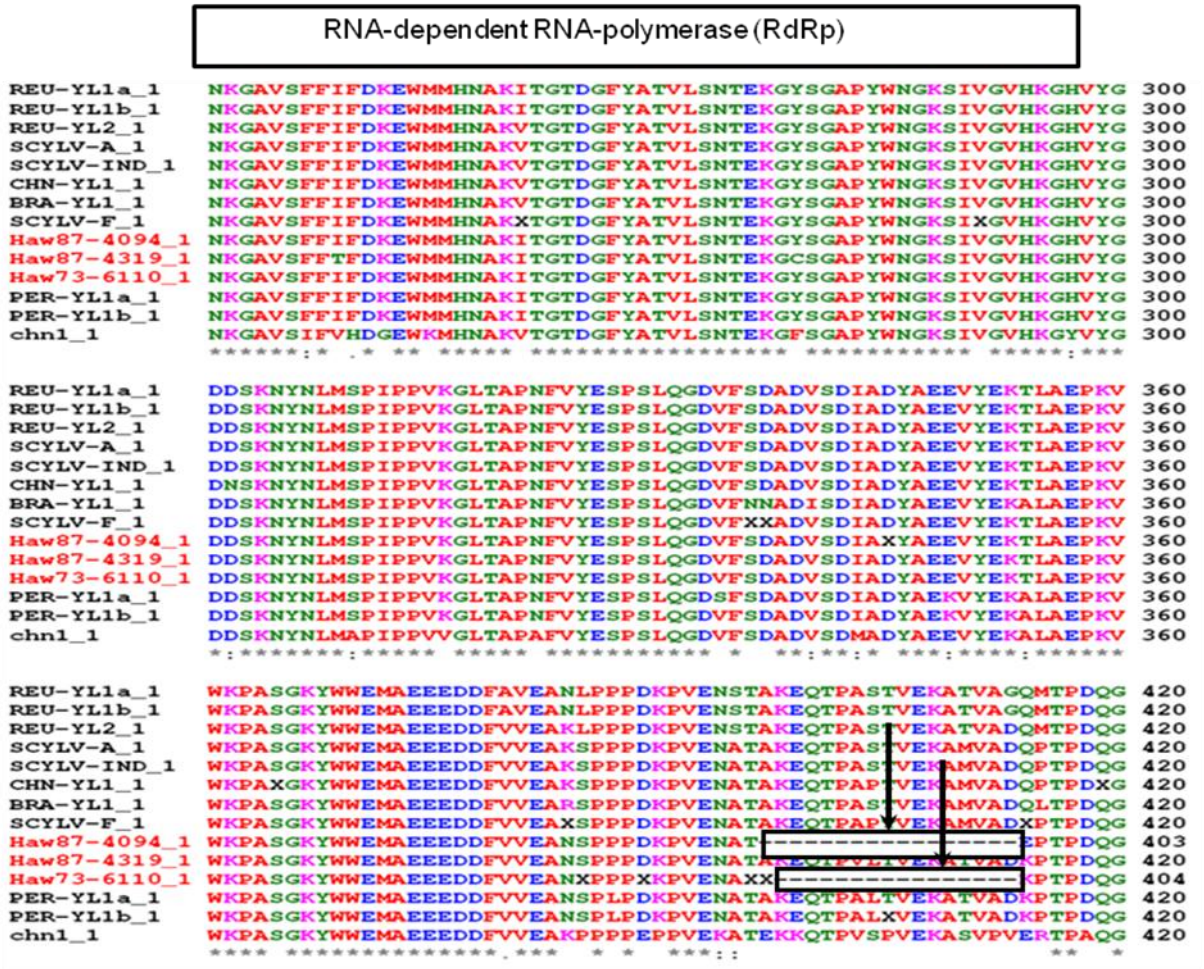


Fig. 6 Alignment of the deduced amino acid sequences of the RdRp region of Hawaiian and several other SCYLV-strains. Asterisks indicate perfect matches with all sequences. Columns denote amino acid differences. The x denotes a place where a nucleotide identity was ambiguous.

Discussion

All tested Hawaiian cultivars contained SCYLV, even the so-called resistant cultivars which previously had been thought to be virus-free based on TBIA (Schenck and Lehrer 2000). Obviously the immunological assay is less sensitive than RT-PCR even for the highly conserved YLS-segment. Only the cultivar line which had been generated by meristem tip culture was indeed virus-free. Semi-quantitative RT-PCR indicated that the susceptible cultivars had a hundredfold higher virus titer than the resistant cultivars. The clone of cultivar H65-7052 grown in the Bayreuth greenhouse had a very low titer which was unexpected because the same cultivar had been previously found to be moderately susceptible, i. e. containing SCYLV at a sufficient concentration to be detected by TBIA, although the level was strongly fluctuating (Lehrer et al. 2007). Either it happened that the RNA-extract from leaves was accidentally made when the fluctuating SCYLV-titer was at minimum or the particular clone in Bayreuth was a low-titer

clone. Recent tests of H65-7052 in Hawaii seemed to indicate that plants of the cultivar H65-7052 with low SCYLV-titer "inherited" the low titer through vegetative seed pieces (Zhu et al., 2010), thus possibly differently proliferate virus strains coexist in this cultivar. We found in northern blots that RNA of Hawaiian SCYLV is divided into genomic RNA and two subgenomic RNAs (Fig. 2) with estimated sizes of 6.0, 2.4 and 1.0 kb, similar to 6.0, 2.4 and 0.8-1.0 kb reported previously (Borth et al., 1994; Moonan et al., 2000). Thus SCYLV may be similar to other plant RNA viruses, which have evolved numerous strategies of genome expression to invade host plants, for example, divided genomes, subgenomic messenger RNAs, frame shifting, overlapping reading frames or stop codon suppression (Zaccomer et al., 1995).

The fragments A-D of SCYLV from Hawaiian cultivars were amplified using the published primer sequences (Abu Ahmad et al. 2006). Not all fragments could be amplified at the same quantity and some fragments from some cultivars were not amplified at all. A similar result was reported by Abu Ahmad et al. (2006). The reasons are unknown but it may be due to sequence divergences in the primer binding regions. The fragment YLS was easily amplified in all SCYLV-preparations and it turned out to be the most conserved region. Three Hawaiian SCYLV-strains isolated from three different cultivars were fully sequenced in this study. We found that the Hawaiian strains (including 3 previously published SCYLV-fragments from Hawaiian cultivars, Haw1-3) are constantly grouped together and located next to or together with strains from Peru, despite some differences. On the whole genome level the PER strains are next to Hawaii isolates and apart from the BRA strains (Fig. 4f). This close relationship may be explained by the fact that the Peru-strain was isolated from sugarcane cultivars, which were developed in Hawaii and exported to Peru, probably already infected with SCYLV in Hawaii. From the phylogenetic analysis we propose that a new SCYLV-group is defined namely a HAW-PER group, or, alternatively, as a subgroup of the BRA-strains, the next relatives to the PER and HAW strains. It would be interesting to analyse the SCYLV-strains from the same cultivars which are currently grown in the Hawaiian breeding station to see, whether the small differences to the Peru-strains were already present in Hawaii or whether they derived from sequence changes or recombinations with BRA strains in the past 30 years in Peru. The REU strains and a recently published genome from a Chinese strain (CHN) represent distinct groups each (Fig. 4f). The phylogenetic distances between SCYLV-strains are insignificant for the fragment YLS, which was already previously called a diagnostic sequence (Comstock et al. 1998). The coat protein encoded by fragment YLS is extremely conserved, only five amino acid difference were detected for all SCYLV-strains where CP deduced amino acid sequence is available. Therefore the immunological test for SCYLV by an antibody directed against the coat protein (Lockhart et

al. 2000) is a valid diagnostic field test for the presence of the virus despite its limiting detection threshold. The CP is directly associated with the success of infection, as it is involved in viral transmission, particle packaging, and viral accumulation within the plant (Peiffer et al., 1997, Brault et al., 2003). Thus, a high degree of conservation in the CP protein sequence is expected. In contrast, the other fragments exhibit phylogenetic distances up to twice as large as fragment YLS. The variations in RNA-sequence and deduced amino acid sequence were found to be relatively high in RNA-dependent RNA-polymerase, as previously reported by Moonan and Mirkov (2002). There is a phylogenetic inconsistency between fragments B and C concerning ScYLV-C1 and –L1, which cluster together in fragment B, but are far apart in fragment C (Fig. 5c and d). Interestingly, the first half of fragment C was derived from *Potato leaf roll virus*, the second half from *Barley yellow dwarf virus* (Moonan et al., 2000). Possibly these two sequence parts may have diverged differently during evolution (or recombination) with the result of an ambiguous. The SCYLV-C1 sequence had been taken previously as evidence for the Colombian strain to belong to a progenitor population of the other SCYLV strains (Moonan and Mirkov, 2002).

Sequence comparison of SCYLV between two susceptible and one resistant cultivars showed a 48 to 54 nt long deletion in SCYLV isolated from susceptible cultivars. This deletion is located in the RNA-dependent RNA-polymerase/silencing suppressor ORF1/2. The RNA-dependent RNA polymerase (RdRp) plays a central role in the replication of RNA viruses and it is tempting to speculate that theses deletions could play a role in controlling the proliferation rates of SCYLV, thereby increasing the SCYLV titre in the susceptible cultivars. Previously, short deletions of 3 and 1 nt in ORF2-3 have been reported in a Colombian strain and in cv. SP71-6163 (Moonan and Mirkov 2002) and a 25 nt deletion in fragment C related to ORF3 (AbuAhmad et al. 2006). These reported gaps are obviously very different in size and position from that found in our case. A similar gap at the same position as in H73-6110 and H87-4094 was found in GenBank entry AJ491131 derived from cultivar CP65-357 (Smith et al. 2000), which is reported to be highly susceptible to YL (Lockhart and Cronje 2000). Amazingly, the nucleotide sequence of many GenBank entries start around the first nucleotides after the deletion, thus it is unknown whether the nucleotides before were absent or were eliminated. Future analysis of other susceptible and resistant cultivars should show whether the susceptibility for SCYLV can be correlated with the absence of the 48 to 54 nt stretches in ORF1/2. The reason for the deletion in the sequence of SCYLV from some cultivars is unknown, it could indicate the presence of two differently proliferate virus strains or a different splicing of the viral RNA by sugarcane cultivars. In the first case one would expect mixed SCYLV-infections of the cultivars,

because the breeding station is highly infested by viruliferous aphids, which should result in a strong interchange of the two (or more) SCYLV-strains. In the second case the splicing of viral RNA by plant spliceosomes needs that the viral RNA has access to the nuclear space or to a cytosolic spliceosome (König et al. 2007). However, the flanking regions of the deleted sequence do not represent a general splicing signal. Future controlled infection experiments with viruliferous aphids may cast more light on SCYLV-susceptibility, whether it is a virus or a plant feature.

Acknowledgement

The help and advice by Dr. Trampczynska and S. Rentsch (Bayreuth, Germany) is gratefully acknowledged. A. Elsayed is grateful to the Egyptian Government for the stipendium. We also thank Bayreuth University for a special fund and Fonds der Chemischen Industrie.

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7. Expression of sucrose transporter (ShSUT1) in a Hawaiian sugarcane cultivar infected with Sugarcane yellow leaf virus (SCYLV)

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Keywords: *Saccharum spec*. hybrid, ShSUT1A transcript, sucrose transporter, *Sugarcane yellow leaf virus*

Abbreviations: cv.: cultivar, ShSUT1A: Saccharum hybrid cultivar sucrose transporter 1A, SCYLV: Sugarcane yellow leaf virus

Submitted to *Physiological and Molecular Plant Pathology*

Abstract: A sugarcane disease was detected in Hawaii in the 1990s, which is caused by the phloem-located *Sugarcane yellow leaf virus* (SCYLV). The sucrose transporter was isolated from a Hawaiian cultivar and its distribution in the plant was determined. The transporter belongs to the SUT1 group. It is expressed in leaves and stem internodes, in the latter the transcript levels increase during maturation and sucrose storage. No significant differences of transcript levels were found between SCYLV-infected and virus-free sugarcane. Therefore the previously reported reduction of assimilate export in SCYLV-infected plants cannot be attributed to a reduction of sucrose transporter expression.

Introduction

In light of the global needs for renewable energy, sugarcane is one of the most productive crops as a source for sugar and bio-fuel. The mature stem can accumulate close to 700 mM sucrose which is in excess of 50% of its dry weight (Moore, 1995). In the past decade a new viral sugarcane disease was detected, Yellow Leaf, caused by the polerovirus *Sugarcane yellow leaf virus*. The disease causes leaf yellowing and significant yield declines, is, however, not lethal. Viral infection often affects carbon assimilation and metabolism in host plants and the onset of leaf symptoms is caused by localised changes in the chloroplast structure and function (van

Kooten et al. 1990 and Yan et al. 2009). Analysis of carbohydrate levels, sugar metabolism and chloroplast structure in SCYLV-infected leaves of Hawaiian sugarcane cultivars led to the conclusion that these changes were secondary effects caused by inhibition of sucrose export from the leaves to the stem (Yan et al. 2009 and Lehrer et al. 2007). A decrease of export may be caused by plugging of sucrose transport paths or by a decrease of sucrose transporters. Source leaves of transgenic tobacco plants expressing the movement protein of Potato leafroll virus (PLRV) showed accumulation of carbohydrates and a decrease in photosynthetic capacity (Herbers et al. 1997). Hofius et al. 2001 proposed a role for cell wall invertase in up-regulating the accumulation of soluble sugars and down-regulating photosynthesis, thus strengthening defense responses against viral attack. The plasmodesmata in the phloem of these plants were altered compared to the wild-type. Plasmodesmata in SCYLV-infected sugarcane were, however, not altered (Yan et al. 2009). A possible reason for decreased sugar export in infected sugarcane may therefore lie in a decrease of sucrose transporter expression.

Sucrose transport in sugarcane can be divided in two parts, the phloem loading of sucrose, mostly in the leaf veins, and the sucrose storage in the stem parenchyma. Principally symplastic and apoplastic routes lead sucrose to the phloem. The phloem conducting cells in sugarcane leaves are not connected to other leaf cells by plasmodesmata (Robinson-Beers and Evert, 1991), so that the phloem loading into the sieve tube-companion cell complex is apoplastic. Movement of sucrose into the leaf bundles through the bundle sheath is most likely symplastic, because the bundle sheath is surrounded by a lignified membrane which probably prevents apoplastic solute flow. A sucrose transporter (ShSUT1) was identified in sugarcane which is expressed in leaves and stems (Rae et al. 2005a). Expression of SUT1-group transporter in yeast mutants has shown that these transporters act as sucrose-proton symporters. The SUT1 subfamily is a high-affinity low-capacity subfamily and has been identified in many plant species as essential for phloem loading. The ShSUT1-protein was localized in the layer of cells surrounding the bundle sheath, but was absent from the sugarcane phloem itself (Rae et al. 2005a and Casu et al. 2003). The sucrose transport in storage tissue is possibly different from phloem loading. Sucrose accumulation in sugarcane is the result of cycling and turnover of sucrose in cytosol and vacuole and apoplastic compartments are possibly in kinetic equilibrium with cytosol (Moore, 1995 and Komor, 2000). Whether these processes are directly affected by SCYLV-infection is unknown, but it is known that the sucrose concentration in infected stems tends to be higher than in un-infected stems (Lehrer et al. 2007).

In this study, we evaluated by RT-PCR and Northern blot the presence of sucrose transporter from a Hawaiian cultivar in leaves and internodes of virus-free and SCYLV-infected sugarcane.

As prerequisite for this study, the sucrose transporter from Hawaiian sugarcane was isolated and characterized, because it was experienced in the past that the large divergence of commercial sugarcane cultivars, caused by incrossings of different *Saccharum* species (*S. officinarum, S. robustum, S. sinensis*), had resulted in significant changes of genotypes between regional sugarcane cultivars with the consequence that published gene sequences obtained from other sugarcane progenies could not be used without proof for molecular studies.

Material and Methods

Plants

The plants were propagated from cuttings of virus free cultivar H87-4094 and of SCYLV-infected cv. H87-4094, both obtained from the Hawaii Agriculture Research Centre (Aiea, Hawaii, USA). The virus-free line of cv. H87-4094 was produced by meristem tip tissue culture and provided by Dr. A. Lehrer (Honolulu, Hawaii, USA). The sugarcane plants were grown in the greenhouse of Bayreuth University (Germany) at 24 ˚C with a 12 h photoperiod. The leaves and internodes were numbered acording breeders´ practice, identifiying the uppermost leaf with fully developed dewlap and the attached internode beneath as #1.

Isolation of RNA from plant tissues

RNA was extracted and purified from plant tissues (source leaf, sink leaf and internodes) according a modified method of Sambrook and Russell 2001. Approximately 200 mg of frozen sample were ground to a fine powder in liquid nitrogen and transferred to a snap-cap tube. RNA extraction buffer (Triton X-100, 100 mM NaCl, 10 mM Tris-HCL, 1 mM EDTA and 1%SDS) and acid phenol-chloroform were used for the extraction. The supernatant was treated with 5 µL of DNAse buffer and 5 µL of DNAse I (1U/µL) were added and incubated at room temperature for 15 min. The washing and centrifugations were done according to Sambrook and Russell 2001. The pellet was air dried and resuspended in 50 µL of DEPC-treated water. The concentration of RNA was determined by Nanophotometer (IMPLEN, Munich, Germany). The preparation was stored at -80˚C.

ShSUT1A cloning and sequencing

The primer for sequencing was designed according to the sequence of GenBank accession number (AY780256). RT-PCR product was cloned with the pGEM®-T Easy Vector System (Promega) using the manufacturer´s protocol, and was transformed into *E. coli* DH5α.The recombinant DNA clones containing the inserts of expected size were purified using the Pure yield™ Plasmid Miniprep System (Promega). The selected clone was sequenced by primer walking using M13 sequencing primers and internal primer of ShSUT1A gene which were designed to sequence the entire clone. The clone was sequenced (DNA Analytics Core Facility at the University of Bayreuth) and used for alignment and phylogenetic analysis.

RT-PCR for expression of ShSUT1A sucrose transporter in sugarcane

RT-PCR was used to determine the expression of ShSUT1A in sink, source and internodes tissues of sugarcane. RT-PCR was performed with primers: Fow. 5´GCTATGCGGTCCTATTGCTG3´and Rev. 5´AGATCTTGGGCAGCAGGAAC3´. The RNA from all samples was reverse transcribed using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, USA), primed with 50 pmol of reverse primer by following the manufacturer's protocol in a PCR machine (PTC 100 Peltier Thermal Cycler, MJ Research, Global Medical Instrumentation, Inc, USA.). The RT- PCR reaction was performed in a volume of 25 µL containing 1 µL cDNA, 2.5 µL of 10x PCR buffer (containing 15 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mix), 10 pmol each of forward and reverse primers for ShSUT1A, 1 unit of polymerase with proofreading activity (*Pfu*) *Taq* polymerase (5:1) (Stratagene, Waldbronn, Germany), and sterile milliQ water added up to the final volume. This PCR programm was performed with initial denaturation at 94°C for 4 min, 10 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, and 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min with a final 72°C extension for 7 min.

A 10 µL aliquot of each amplified product was analysed by electrophoresis on 1% agarose gels stained with ethidium bromide to confirm the presence of a PCR product of the expected size. The RT-PCR was performed with internal control, housekeeping (25S rRNA) as a reference gene to normalize patterns of gene expression and evaluate the integrity of cDNA. Furthermore, the primer sets were optimized using semi qPCR with different numbers of PCR cycles.

Northern blot analysis

Ten micrograms of intact RNA isolated from sugarcane leaves, seedling and internodes were fractionated on a 1.2% formaldehyde-agarose gel in MOPS buffer run at 80V for 2 h. The gel was stained with ethidium bromide and photographs were taken. Downward blotting was set up to transfer mRNA to a positively charged nylon membrane (0.45-µm pore size, Hybond N+, Amersham GE Healthcare Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, UK), by capillary transfer using 20xSSC for 16 h. Thereafter the membrane was UV crosslinked for 1 min and heat-treated at 80 °C for 2h. The membrane was briefly washed in 5xSSC for 1 min at room temperature. The membrane was transferred to hybridization tube with 30 mL of prehybridization buffer and incubated for 1 h at 68 °C under gentle agitation. The RNA probe of ShSUT1A was produced by using PCR generated templates for in vitro transcription. The DNA fragment was amplified by RT-PCR and cloned into pGEM®-T (Promega, Mannheim, Germany). The orientation of the insert fragment was determined by sequencing. The transcription of RNA anti-sense probe and the hybridization were performed as described in the DIG System Users Guide (Roche Diagnostics GmbH, Mannheim, Germany). The blot was labelled with anti-digoxigenin-AP and chemiluminescent detection (CDP-Star ready-to-use) and visualized with a chemilux CCD camera (Intas, Göttingen-Germany).

In Situ Hybridization

Plant material was fixed with 4% paraformaldehyde (Sigma-Aldrich, Munich, Germany) in PBS for 8 hr after vacuum infilteration. The tissue was dehydrated and embedded in Paraplast X-tra (Sigma-Aldrich, Munich, Germany). Eight-micrometer sections were placed on SuperForst/Plus slides (Menzel Gläser). Paraplast was removed by immersion in Histoclear. Sections were rehydrated, incubated 10 min 0.125mg/ml Pronase (Sigma-Aldrich, Munich, Germany) in TE (50mM Tris-HCl pH 7.5, 5mM EDTA), 10 min in 4% paraformaldehyde in PBS, and 10 min in 0.5% acetic anhydride in 0.1 M triethanolamine (pH 8). After dehydration by an ethanol series, slides were air dried before application of the hybridization solution. Per slide, 50-200 ng probe was applied in 80 µl hybridization solution. After incubation in a humid box at 50ºC overnight, slides were washed twice in 0.2x SSC for 1 hr at 55ºC. After incubation with 20 µg/ml RNAse A for 20 min at 37ºC, slides were again washed in 0.2x SSC for 1 hr at 55ºC. Slides were incubation in 0.5% blocking reagent (Boehringer, Penzberg) in TBS (100 mM Tris [pH 7.5], 150 mM NaCl) and gently agitated for 45 min. Anti-Digoxigenin-alkaline-phosphatase-coupled antibody (Boehringer) was diluted 1:1250 in BXT (1% BSA, 0.3% Triton X-100 in TBS), 120 µl applied to each slide with a cover slip, and incubated for 2 hr. Slides were then washed 4x 20

min with BXT. One hundred microliters of fresh staining solution (220 µg/ml NBT and 80 µg/ml BCIP in 100 mM Tris [pH 9.5], 50 mM MgCl2, 100mM NaCl) and cover slips were applied daily for 14-18 hr. for microscopy, 50% glycerol and a cover slip were applied. Photographs were made using a Zeiss Axioskop 2 plus with Zeiss Axiocam MRc camera. Probes were labelled using Digoxigenin labelling mix (Boehringer) according to the manufacturer's protocol. An antisense probe from ShSUT1A cDNA clone was generated using T7 RNA polymerase, and a sense probe was synthesized using SP6 RNA polymerase. An antisense probe from SCYLV cDNA clone was generated using SP6 RNA polymerase, and a sense probe was synthesized using T7 RNA polymerase.

Alignment of sequences and construction of phylogenetic trees

Multiple sequence alignments of nucleotide or deduced amino acid sequences were aligned using CLUSTAL W applying the Dayhoff PAM 250 matrix (Thompson et al. 1994) and were optimized manually. Phylogenetic reconstructions were performed using Geneious program, version 4.7.5 (www.geneious.com). Trees were constructed by the UPGMA method. Data sets were bootstrapped (1,000 replicates) to assess the confidence values of the phylogenetic trees, and bootstrap values < 50% were omitted. The resulting sequences were compared with the GenBank database (NCBI).

High performance thin layer chromatography (HPTLC) of sugars

Ten milligram of freeze dried samples were extracted with 5 mL of distilled water and co-extracted protein was precipitated with 2 mL of cold acetone. The extract was filtered and adjusted to 10 mL with methanol. Five microliters of sugar extract as well as 5 µL standard material (sucrose, fructose and glucose) were applied using CAMAG (CAMAG, Muttenz, Swizerland) automatic TLC sampler on TLC plates (Silica gel F_{254}, 10 x 20 cm, thickness 0.25 mm, Merck, Darmstadt, Germany). Samples were developed in CAMAG Automated Multiple Development (AMD) using the solvent system containing acetonitrile/water (85:15, v/v) wherein the migration distance was 70 mm. For the detection of sugars, the thin-layer plates were sprayed with diphenylamine reagent followed by heating at about 120 °C for 10 min. with CAMAG TLC scanner and CATS software, individual bands were visualized under ultraviolet light and scaned by absorbance at 620 nm.

Results

In situ analysis of SCYLV in sugarcane stem and leaf

Routine analysis for SCYLV is performed by either tissue blot immunoassay, in which a leaf midrib is printed on membrane and the blot is then developed with antibody, or by RNA-extracts of leaves. In situ hybridization of leaves had shown that SCYLV is confined to the phloem (Yan et al. 2009). Tissue blots of stems and RNA extracts from the extremely sugar-rich internodes are difficult to obtain. Therefore the presence of SCYLV in stem internodes was only suspected so far but not shown. Storage parenchyma of maturing internodes was shown to be symplastically connected to the internode phloem (Jacobsen et al. 1992) so that a transfer of SCYLV infection from bundles into parenchyma cells could be imagined. In situ hybridization with SCYLV-antisense probe, however, confirmed that SCYLV is confined to the companion cells of the internode phloem (Fig. 1). SCYLV transcript levels appeared to be higher in mature internode (#8) than in internode #5. In control experiments with a sense RNA probe, only weak background colour was developed (Fig.1D).

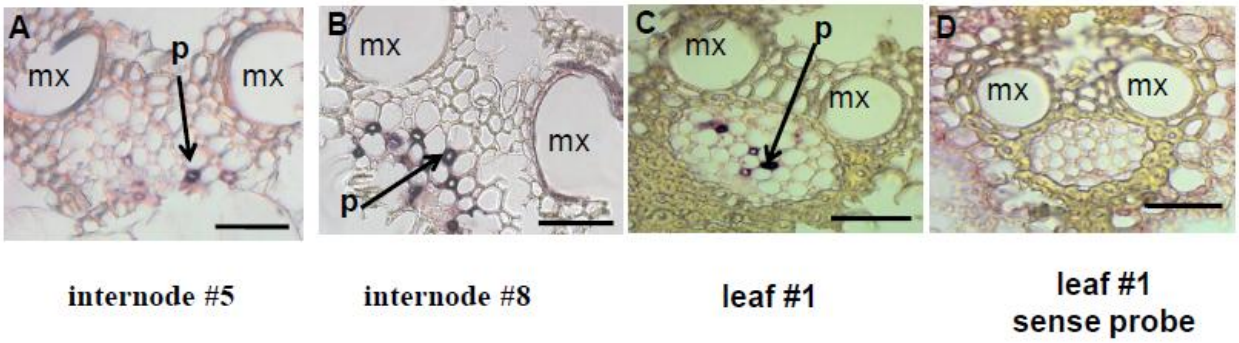


Fig. 1 *Localization of SCYLV by in situ hybridization on sections of sugarcane leaf (#1) and stem (internodes #5 and #8).* The sections were hybridized with the antisense probe (A-C) or a sense probe (D). (mx: metaxylem; p: phloem companion cell; bar equals 100 µm).

Isolation of ShSUT1A and its phylogenetic relationship to the SUT sucrose transporters

ShSUT1A from Hawaiian cv. H87-4094 was isolated using primers deduced from the published sequences (Casu et al. 2003). The amino acid sequence of the isolated transporter showed 97.1% sequence identity with ShSUT1. A major difference was a seven amino acids stretch at position 203- 210 in the isolated clone which was absent in the published sugarcane sequence ShSUT1 and in other SUT1 sequences (Fig. 2). We therefore called the sequence ShSUT1A to

differentiate it from the published transporter ShSUT1. An alignment with other SUT1 sequences from 8 grass species and a few dicot species was performed by standard software. The alignment revealed a monocot-specific box of 5 amino acids at position 60-65 (Fig. 2) which is part of the loop between transmembrane helices 1 and 2. The dicot SUT1 were characterized by a 2 amino acid stretch after position 159 and, in some cases, a 5 amino acid stretch at around position 373.

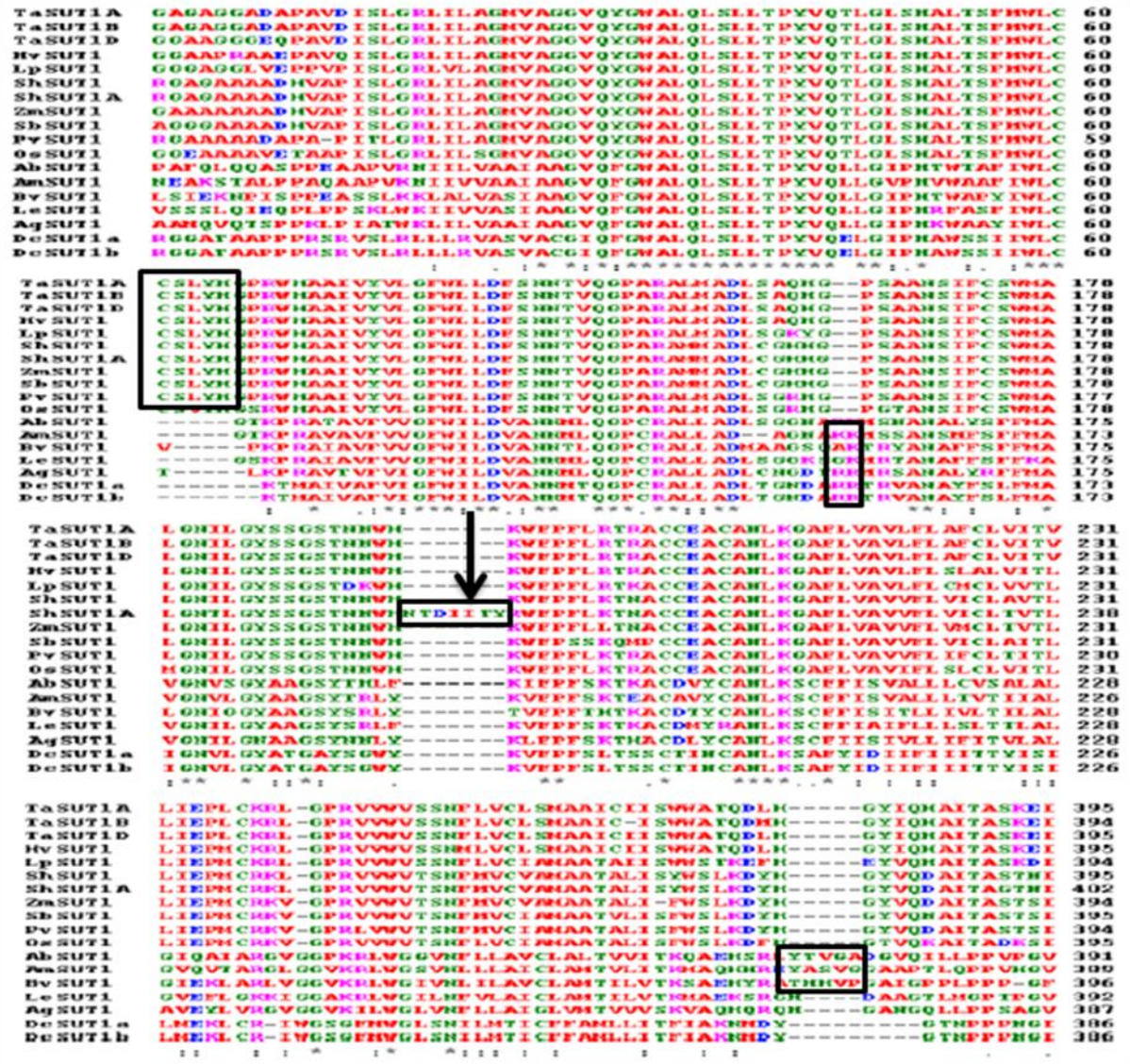


Fig. 2 *Alignment of the deduced amino acid sequence of the sucrose transporter gene ShSUT1A with published SUT1 sucrose transporters.* Asteriks indicate perfect matches within all sequences. Black boxes denote residues that were identified as different regions and absent in some sequences. The sequences were aligned using CLUSTAL W applying the Dayhoff PAM 250 matrix (Thompson et al. 1994). The sequences are: TaSUT1A-D from *Triticum aestivum*, HvSUT1 from *Hordeum vulgare*, LpSUT1 from *Lolium perenne*, ShSUT1,A from *Saccharum spec.* hybrid, ZmSUT1 from *Zea mays*, SbSUT1 from *Sorghum bicolor*, PvSUT1 from *Panicum virgatum*, OsSUT1 from *Oryza sativa*, AbSUT1 from *Asarina barclaiana*, AmSUT1 from *Alonsona meridionals*, BvSUT1 from *Beta vulgaris*, LeSUT1 from *Lycopersicon esculentum*, AgSUT1 from *Apium graveolens*, and DcSUT1a,b from *Daucus carota*. Accession numbers see legend to Fig. 3.

The taxonomic position of ShSUT1A within the sucrose transporters was analysed in comparison with 34 nucleotide sequences of other, well-characterized SUT members. Three major groups showed up **(**Fig. 3): Cluster 1 contained 17 SUT sequences from monocot (*Oryza sativa*, *Hordeum vulgare)* and many dicot species (*Apium graveolens*, *Asarina barclaiana*, *Beta vulgaris*, *Daucus carota*, *Arabidopsis thaliana*, *Lycopersicon esculentum*, *Nicotiana tabacum*, , *Lotus japonicas* and *Pisum sativum)* covering SUT1-5. The similarities among sequences were varied 73 -100%. Cluster 1 also included dicot SUT4 (LeSUT4 and LjSUT4, bootstrap of 73%). Exclusively monocot SUT were assembled in cluster 2, including 13 SUT1s with high bootstrap from 83 to 100%. ShSUT1A was closest to ShSUT1 with 99.9% identity. OsSUT3 and OsSUT5 were relatively close to monocot SUT1 and appeared in between of cluster 2 and 3, which was formed by sucrose transporters of dicot SUT2 (AtSUC3, LeSUT2) and OsSUT4, exhibiting 78.3% bootstrap value with the monocot SUT1. AtSUT4 was relatively separated from the other sucrose transporters.

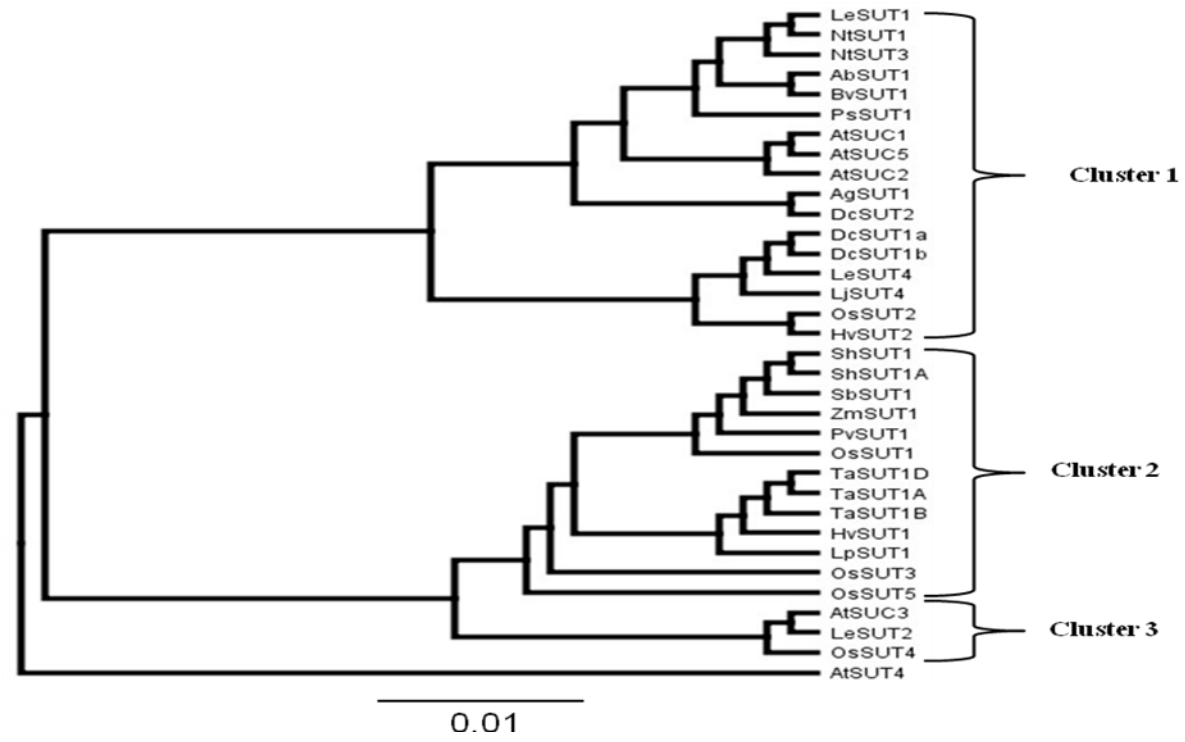


Fig. 3 *Phylogenetic tree constructed based on nucleotide sequence alignments of 34 plant sucrose transporter (SUTs) and position of sugarcane sucrose transporter ShSUT1A (arrow).* The tree was constructed with Geneious program and UPGMA method and aligned with CLUSTAL W. Accession numbers of presented sucrose transporter sequences are: AbSUT1 (*Asarina barclaiana;* AF191024), AgSUT1 (*Apium graveolens*; AF063400), AmSUT1 (*Alonsona meridionals*; AF191025), AtSUC1 (*Arabidopsis thaliana*; X75365), AtSUC2 (*Arabidopsis thaliana*; X75382), AtSUC3 (*Arabidopsis thaliana*; AJ289165), AtSUT4 (*Arabidopsis thaliana*; AF175321), AtSUC5 (*Arabidopsis thaliana*; AJ252133), BvSUT1 (*Beta vulgaris*; U64967), DcSUT1a (*Daucus carota*; Y16766), DcSUT1b (*Daucus*

carota; Y16767), DcSUT2 (*Daucus carota*; Y16768), HvSUT1 (*Hordeum vulgare*; AJ272309), HvSUT2 (*Hordeum vulgare*; AJ272308), LeSUT1 (*Lycopersicon esculentum*; X82275), LeSUT2 (*Lycopersicon esculentum*; AF166498), LeSUT4 (*Lycopersicon esculentum*; AF176950), LjSUT4 (*Lotus japonicas*; AJ538041), LpSUT1 (*Lolium perenne*; EU255258),NtSUT1 (*Nicotiana tabacum*; X82276), NtSUT3 (*Nicotiana tabacum*; AF149981), OsSUT1 (*Oryza sativa*; D87819), OsSUT2 (*Oryza sativa*; AB091672), OsSUT3 (*Oryza sativa*; AB071809), OsSUT4 (*Oryza sativa*; AB091673), PsSUT1 (*Pisum sativum*; AF109922),PvSUT1 (*Panicum virgatum*; FJ839440), SbSUT1 (*Sorghum bicolor*; XM_002467230), ShSUT1 (*Saccharum* hybrid; AY780256), ShSUT1A (*Saccharum* hybrid; GU812864), TaSUT1A (*Triticum aestivum*; AF408842), TaSUT1B (*Triticum aestivum*; AF408843), TaSUT1D (*Triticum aestivum*; AF408844) ZmSUT1 (*Zea mays*; AB008464).

The alignment of deduced amino acid residues of ShSUT1 and ShSUT1A show very similar distribution of the putative transmembrane helices, the extra sequence part of 7 amino acids at position 203- 210 did not cause any apparent change of protein secondary structure, but the loop between transmembrane helix 5 and 6 is a bit longer (data not shown).

Expression of sucrose transporter ShSUT1A in different tissues of sugarcane

The expression of sucrose transporter ShSUT1A was detected by RT-PCR and Northern blot in sink leaves, source leaves and internode storage tissue of SCYLV-infected (Vinf) and virus-free plants of cv. H87-4094 (Vf). It appears from RT-PCR that ShSUT1A is slightly higher expressed in the leaves of the infected plants compared to virus free plants (Fig.4a). A strong expression of ShSUT1A appears in seedling shoots, too. The hybridization of RNA with specific probe also showed that ShSUT1A was slightly higher expressed in the leaves of the infected plant than in the virus free plant (Fig. 4b). The transcript expression in the seedling shoot was strong in both virus free and infected plants.

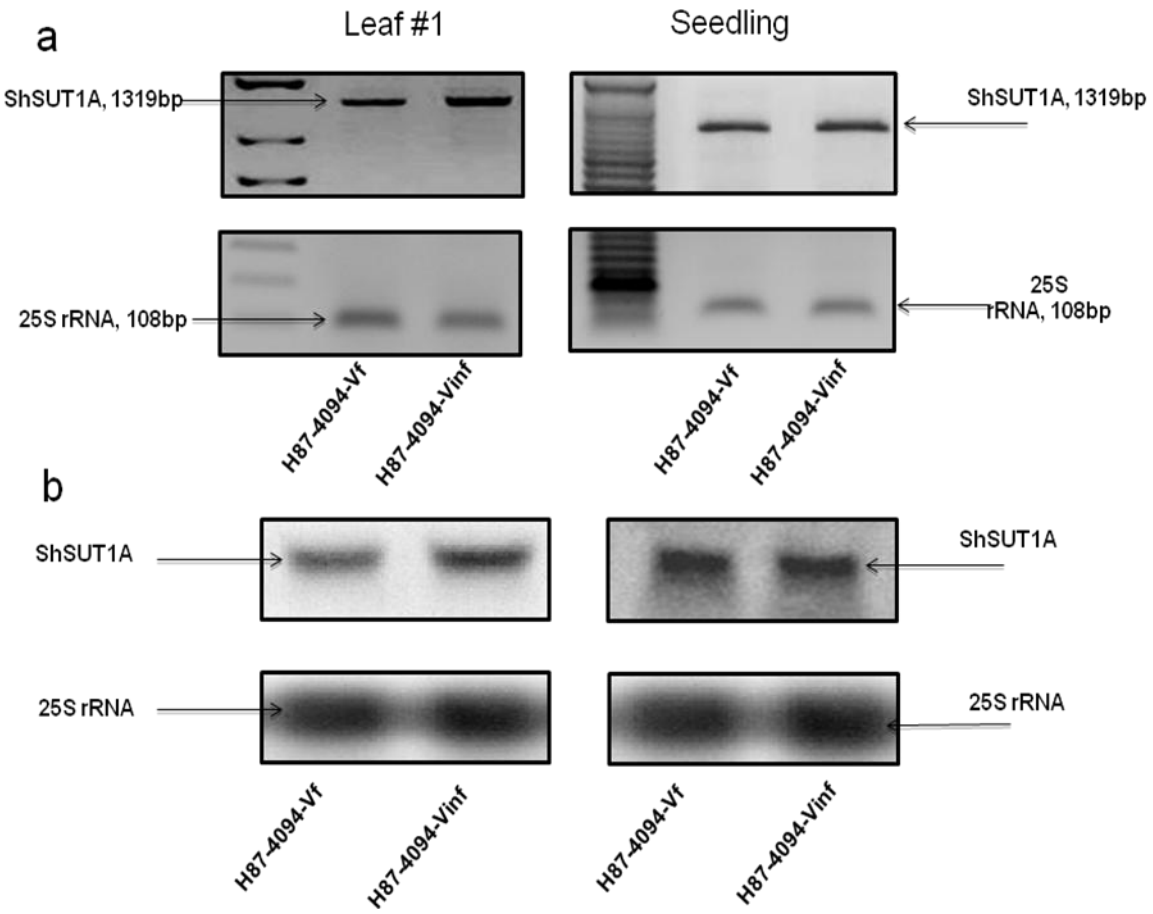


Fig. 4 *Expression of ShSUT1A using RT-PCR (a) and Northern blot (b) in sugarcane leaves and seedling shoost of virus free (Vf) and infected (Vinf) plants of cv. H87-4094.* The lower panels in a and b show the transcription of ribosomal RNA as control for cDNA amplification (25SrRNA, 108bp) or as loading control, respectively. 50bp and 1kb DNA (Fermentas, St. Leon Rot, Germany) were used as molecular size markers.

The expression of ShSUT1A in the intermodal tissues tested by RT-PCR showed a progressive increase from internode number 1 (immature) to 7 (mature) (Fig. 5). Again, the expression of ShSUT1A in the internodes of virus free sugarcane appeared to be less than in the infected plants. The hybridization of RNA (Northern blot) also showed the progressively stronger expression with ripening of the internodes, but there was no clear difference between infected and virus-free plants (Fig. 6). The increase in transcript level in internodes was concomitant with the increase of sucrose content in the stem, whereas the hexose level stayed relatively the same in all internodes and was low compared to sucrose (Fig. 7).

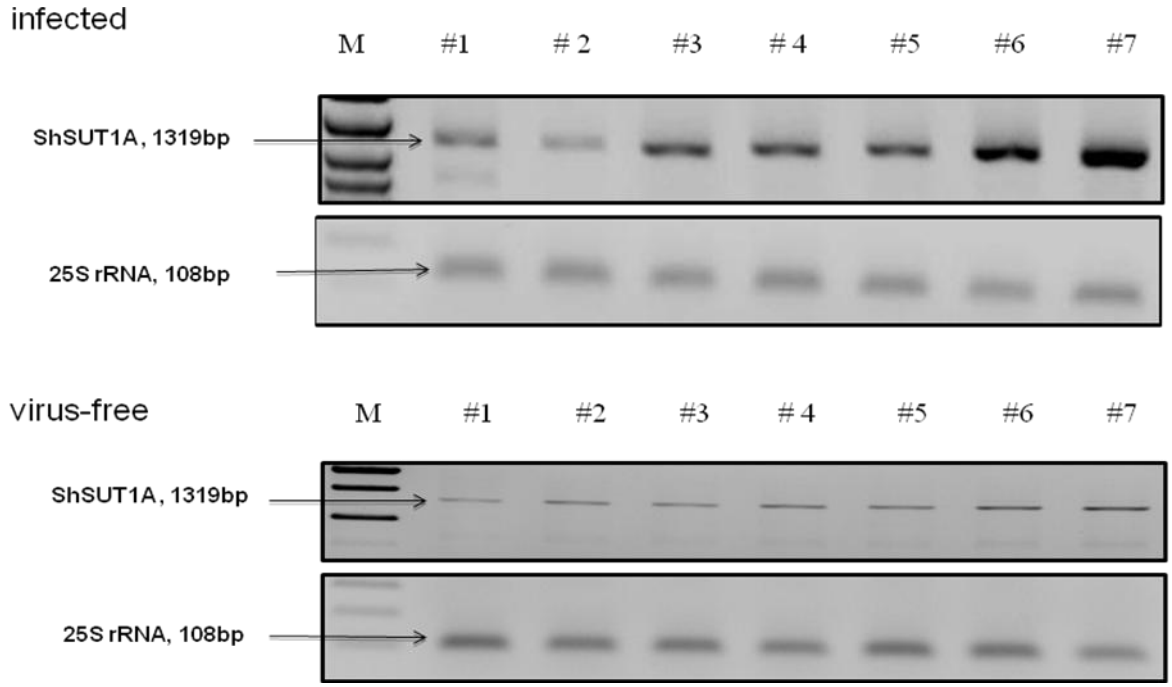


Fig. 5 *RT-PCR for sucrose transporter* ShSUT1A *in internodes (storage tissues) of SCYLV-infected or virus-free sugarcane cv. H87-4094.* Numbers identify the internodes from #1 (immature) to #7 (mature) according their position on the stem. M: DNA molecular size markers 50bp and 1kb (Fermentas, St. Leon Rot, Germany). The lower panel shows the transcription of ribosomal RNA (25SrRNA 108bp) as transcription and amplification control.

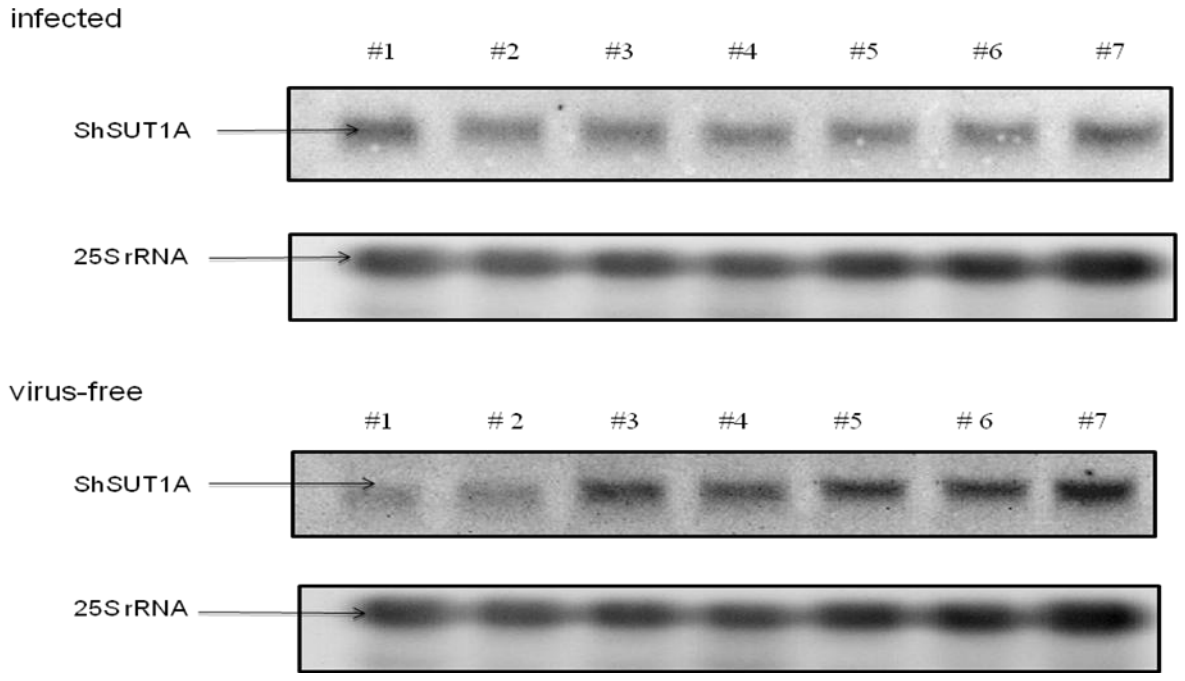


Fig. 6 *Northern blots of transcripts of* ShSUT1A *in in internodes of infected and virus-free sugarcane cv. H87-4094.* RNA was extracted from internodes #1 (beneath first dewlap leaf, immature) to #7 (mature) and was hybridized to a probe of the *ShSUT1A* cDNA. The lower panel shows the same membrane probed for ribosomal RNA as loading control.

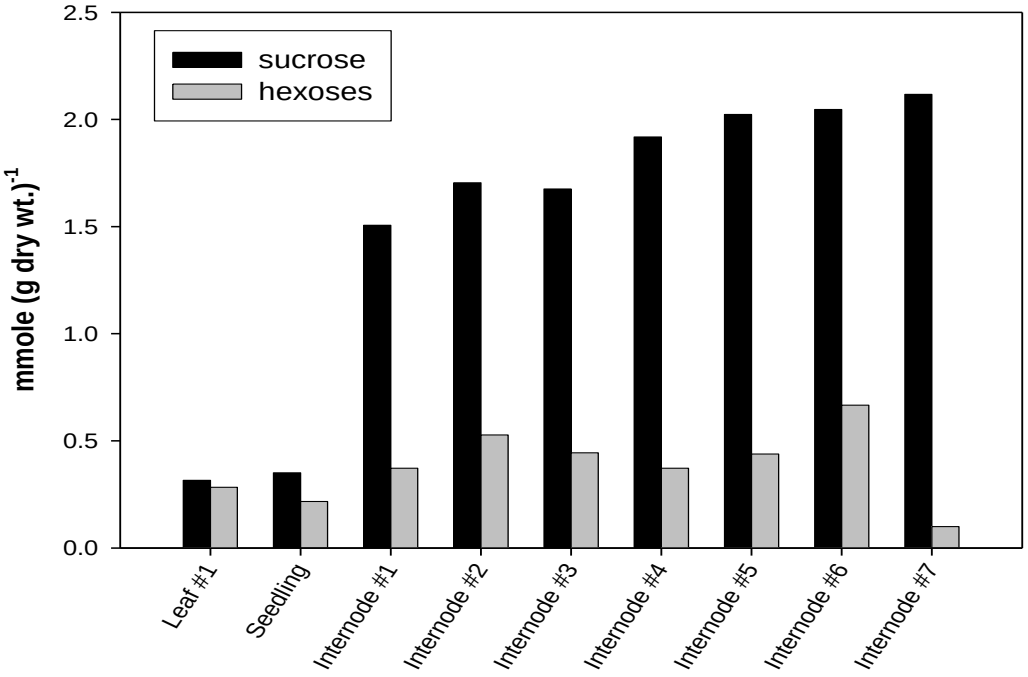
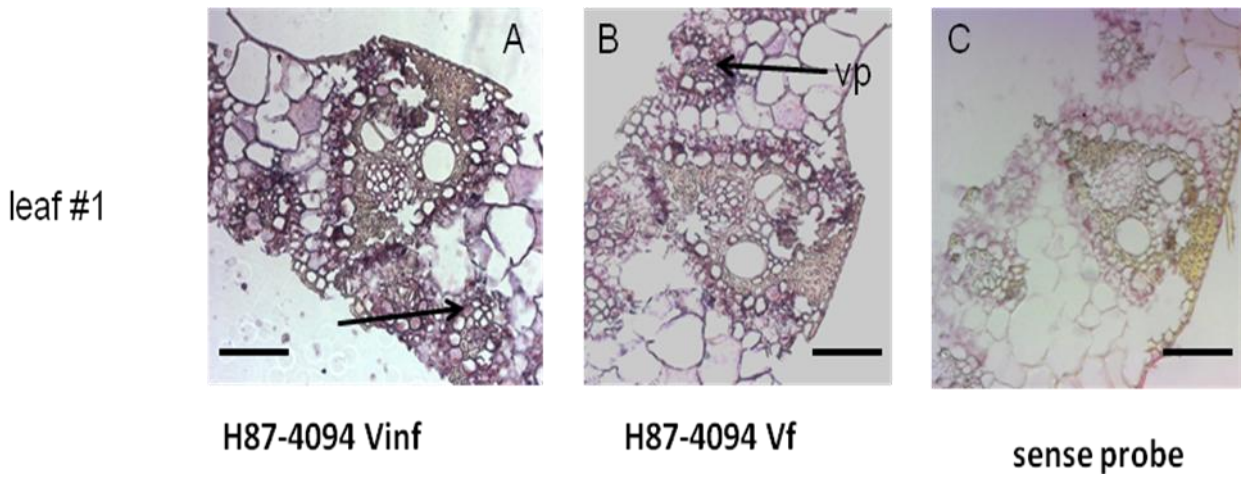


Fig. 7 *Sucrose and hexose content in leaves (#1), seedling shoots and internodes (#1-7) of sugarcane plants(cv. H87-4094).* The sugars were extracted, separated and quantified by HPTLC. The results are the mean of 3 replicates.

In situ localization of ShSUT1A in leaves and internodes (Fig. 8) showed a relatively weak expression in phloem and at the bundle sheath boundaries. No label was seen in the storage parenchyma, however the hybridization quality did not allow the detection of possibly faint signals.



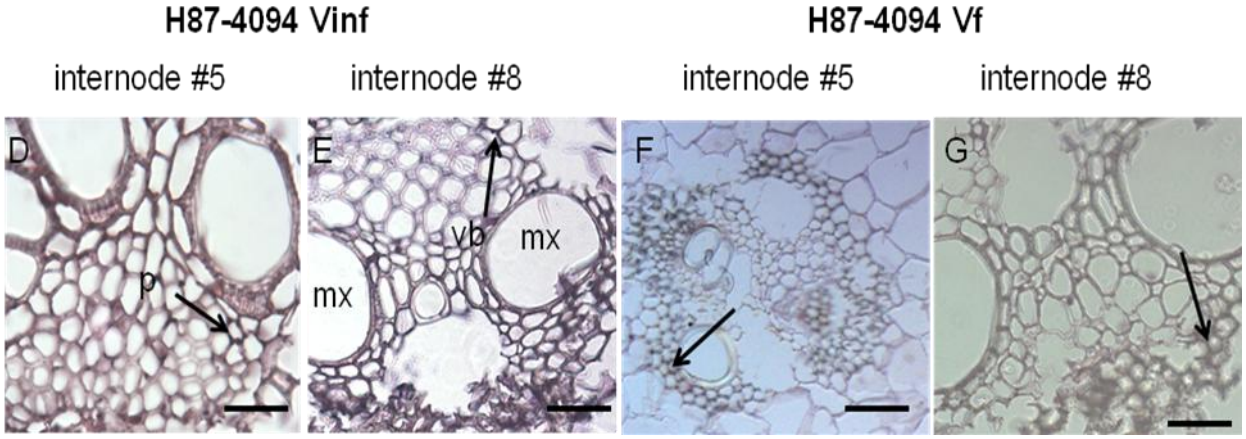


Fig. 8 *In situ localization of ShSUT1A in sugarcane leaf and internodes (#5 and #8) in virus free (Vf) and infected (Vinf) plant.* The sections were hybridized with antisense probe (A, B, D-E) or sense probe (C). Faint transcript signals were present in the vascular parenchyma (vp) of leaves and in phloem (p) and vascular bundles (vb) of internodes. (Mx: metaxylem; bar equals 100 µm)

Discussion

Since the first isolation of a sucrose transporter gene from spinach (Riesmeier et al., 1992), sucrose transporter genes were cloned and sequenced from several species including sugarcane (Casu et al. 2003). Three subfamilies, SUT1, SUT2 and SUT4 were clustered by phylogenetic analysis (data not shown), whereby the SUT1 subfamily is the largest subfamily with high similarity between their members. Despite of these similarities (83-100%), the monocot SUT1 cluster can be separated from a dicot cluster when the amino acid sequences are compared (data not shown). We cloned and sequenced the sucrose transporter from a Hawaiian sugarcane cultivar because there were indications that sugarcane cultivars of one region may be relatively different from cultivars of another regions due to the species-bridging crosses made in the past breeding efforts. The sequence obtained was indeed different from the previously published sequence from an Australian cultivar, especially obvious in the additional stretch of 7 amino acids after the 5th transmembrane domain. Modelling of the molecule by standard software (http://minnou.cchmc.org) did however not reveal a major change in the three-dimensional structure compared to the previously published sequence (data not shown). Still, we felt justified to give the sequence an own name, ShSUT1A, to distinguish it from ShSUT1 obtained from an Australian sugarcane cultivar.

Transcripts of the sucrose transporter were found in all tested tissues, source leaf (leaf #1), sink leaves of a seedling, very immature internodes (internode # 1-3), maturing internodes (internodes #4-6) and mature internodes. The amount of transcripts seemed to increase with maturation (at

least up to internode #7). That is in parallel with the increase of sucrose in the internodes and in parallel with the previously reported increase of sucrose transport into the storage parenchyma cells at the expense of hexose transport (Komor 2000 and Riesmeier et al. 1992). However, possibly sucrose transport and storage in maturing internodes becomes progressively symplastic in parallel with the maturation process, in which a lignified barrier is developed around the bundle sheath, preventing apoplastic transfer from phloem to storage parenchyma (Jacobsen et al. 1992 and Rae et al. 2005b). In addition, the cytoplasmic membrane seems to loose its barrier function with maturation leading to very high apoplastic sugar concentrations, possibly in equilibrium with the cytosolic and vacuolar sucrose concentration (Hawker 1965 and Welbaum et al. 1992). Rae et al. 2005a and b localized ShSUT1 predominantly in the bundle sheath, not in the phloem. Our in situ studies localize ShSUT1A in the phloem of leaves and, although with weak signal, in the phloem of internode bundles. Regrettably the hybridization was too weak to decide whether ShSUT1A is also present in storage parenchyma. The role of sucrose transporters in phloem loading are well documented including in retrieval of sucrose along the transport path in the stem. The localization of ShSUT1A in phloem is therefore no exception, its role in storing sucrose in the parenchyma is less clear. But, whatever the role of ShSUT1, there is definitely no large difference in its transcript levels between SCYLV-infected and virus-free plants of the cultivar, possibly there is a slightly higher transcript level in infected plants. Therefore, the previous observations that SCYLV-infected plants seem to suffer under assimilate export inhibition, cannot be traced back to a lower expression of sucrose transporter SUT1 in source leaves. SCYLV-infected internodes, which definitely contain the virus in the companion cells of the bundles, also seem to contain a higher transcript level than internodes of virus-free plants, which at first sight would be conform with the slightly higher sucrose levels in stems of infected plants (Lehre et al. 2007). However a higher sucrose level may also result from premature maturation because of virus-caused inhibition apical growth, which occurs when infected plants turn symptomatic (Lehrer and Komor 2008). Deficiencies in sucrose transporter expression (assuming that the transcript levels mirror the protein levels) is not the cause for decreased assimilate export in infected plants The remaining alternatives are either a mechanical plugging of the sieve tubes of diseased plants by necrotic callose formation, as is the case in BYDV-infected cereals (Esau, 1956), or a leak of turgor in the companion cell-sieve tube complex because of expression of viral movement protein. Viral movement protein increases the size exclusion limit of plasmodesmata and may thus inhibit phloem loading as was postulated for transgenic plants expressing movement protein of potato leafroll luteovirus (Herbers et al. 1997). However, there may be also indirect effects of viral infection on metabolism or growth such as

inhibiting sugar transport proteins, sugar signalling or metabolic network regulation (Hofius et al. 2001 and Zhang et al. 2009).

Acknowledgements

The help and advice by Dr. S. Clemens and D. Peisker (University Bayreuth) is gratefully acknowledged, also the hospitality of Dr. Thomas Laux and Steffen Knauer for help in in situ analysis (University Freiburg). A. ElSayed is grateful to the Egyptian Government for the stipendium. We also thank Bayreuth University for a special fund.

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8. Simultaneous quantitative analysis of transcripts for *Sugarcane yellow leaf virus,* sucrose transporters and sucrose phosphate synthase in Hawaiian sugarcane cultivars by multiplex RT-PCR

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Keywords: GeXPS multiplex PCR, sucrose phosphate synthase II, sucrose transporters, *Sugarcane yellow leaf virus.*

Submitted to *Plant Pathology*

Abstract: The transcript levels of the open reading frames (ORFs) 0-5 of the *Sugarcane yellow leaf virus* (SCYLV) genome were determined in sink leaves, source leaves and mature internodes of four Hawaiian sugarcane cultivars by GeXP multiplex RT-PCR. The cultivars had been classified previously as SCYLV-susceptible or SCYLV-resistant, a virus-free line was used as control. The transcript levels were normalized to 25S rRNA transcript levels. The transcripts of the ORFs were present in very different quantities in the tested tissues and cultivars. ORF0-1 coding for silencing/suppressor protein had the highest transcript level, ORF3-4 coding for the capsid protein the lowest. The ratio of ORF-transcripts was not constant, neither between tissue types nor between cultivars. Therefore a forged RNA-amplification by the method appeared to be an unlikely reason for the different amplificate quantities. The transcript levels of all ORFs were higher in sink leaves than source leaves and in these higher than in mature internodes.
Quantitative multiplex RT-PCR was used to determine the transcript levels of genes which are important for sucrose storage in sugarcane, namely sucrose phosphate synthase (SPS), sucrose transporter SUT1 and sucrose transporter SUT4. Transcript levels for SPS and of SUT4 were constant throughout the tested tissues and cultivars, SUT1 was highest in sink leaves and lowest in internodes. Consequently SUT4 transcripts appeared to be increasingly important in the sucrose storage process. No consistent differences between the susceptible and the resistant cultivars and no differences between SCYLV-infected and virus-free plants were observed with respect to SPS and sucrose transporter transcripts.

Introduction

The sugarcane disease Yellow leaf was first noticed in Hawaiian plantations (Schenck, 1990) and shortly later in many sugarcane regions of the world (summarized in Komor et al. 2010). The search for the causal agent pointed to a RNA virus (Borth et al. 1994) which was then identified as a luteovirus and named *Sugarcane yellow leaf virus (*SCYLV*)* (Vega et al. 1997, Maia et al. 2000). The sequence revealed that it had obviously evolved as a recombination product of *Barley yellow dwarf virus*, *Potato leaf roll virus* and *Pea enation virus* (Moonan et al. 2000 and Smith et al. 2000) and it was then classified as a member of the polerovirus group. The RNA contains six open reading frames (ORFs) which were assigned to putative functions. Analysis of SCYLV from North, Central and South American sources by comparison of partial sequences and RFLP uncovered intraspecific, regional variation and genotype diversity (Moonan and Mirkov, 2002). A worldwide survey and analysis of SCYLV strains (Abu Ahmad et al. 2006 finally identified 3 SCYLV genotypes, one typical for La Réunion (REU), one for South America (BRA-PER), and one for Cuba (CUB). Hawaiian isolates were not among this study. The genotypes were differently infectuous and virulent in their ability to evoke disease symptoms (Abu Ahmad et al. 2007), for example the BRA strain caused much stronger symptoms than the REU strain. A survey of Hawaiian sugarcane cultivars for SCYLV by tissue blot immunoassay had shown that the majority of commercial cultivars contained the virus, however a definite number of cultivars appeared SCYLV-free (Schenck and Lehrer, 2000). Since the plantations and the breeding station is heavily infested by viruliferous aphids, which would distribute the viral infection to all plants within short time, it was assumed that the SCYLV-free cultivars were SCYLV-resistant in contrast to the other, the susceptible cultivars. Recent analysis with quantitative real time RT-PCR detected SCYLV in the resistant cultivars, too, however at a 10-100 fold lower virus titre, which was apparently below the sensitivity threshold of the immunological assay and therefore not detected in the previous screenings (Zhu et al. 2010). This quantitative analysis had used a sequence part from ORF3-4, the so-called YLS-segment which had been proposed to be a “diagnostic” part of the SCYLV-genome (Comstock et al. 1998). SCYLV from two susceptible and one resistant Hawaiian cultivar were isolated and sequenced (ElSayed et al. submitted). The nucleotide sequence and the deduced amino acid sequence classified these Hawaiian isolates as a separate genotype, tentatively called HAW-PER, next to the BRA genotype. The sequences of SCYLV from the two susceptible cultivars were unique in the sense that they lacked a 48-54 nt stretch in ORF1 which was present in SCYLV from the resistant cultivar. ORF1 putatively codes for a suppressor/RNA-dependent RNA polymerase. The sequence parts which were amplified to yield the whole viral genome were

obtained in very different quantities, however, and in some cases no amplificates were obtained at all. Thus the complete SCYLV sequences from several Hawaiian cultivars could not be analysed in a first attempt. This failure was blamed on possibly small sequence variations in some isolates which might have weakened the binding efficiencies of the primers used. We wanted to know, if the variation in getting amplificates of certain viral genome parts were because of inaccuracy of primers or a due property of the viral RNA. Therefore different viral genome parts out of Hawaiian sugarcane cultivars from which SCYLV was successfully sequenced were quantitatively determined in a multiplex RT-PCR with primers designed for the appropriate sequence parts. In the past all SCYLV isolates were obtained from the uppermost fully-developed source leaf. In a current study we also isolated SCYLV from other plant parts to see, whether the different quantities in amplificate were the same throughout the plant or different for certain plant organs. Also interesting was, whether the virus strain with the 48-54nt longer genome from a so-called resistant cultivar showed another quantitative pattern of transcripts than the shorter genome strain from a susceptible cultivar. The GenomeLab GeXP Genetic Analysis System from Beckman Coulter offers multiplexed, quantitative gene expression analysis capable of examining up to 30 genes in a single reaction from as little as 5 ng total RNA. In this study we determined four different genes of the SCYLV genome related to specific open reading frames (ORF0-1, ORF2, ORF3-4 and ORF5) from the Hawaiian *Sugarcane yellow leaf virus* isolates.

A backup of carbohydrates, mostly starch, had been observed in source leaves of infected plants in previous studies, which led to the conclusion that assimilate export is inhibited by SCYLV-infection (Lehrer et al. 2008). Therefore the sucrose transporter and the SPS transcripts were compared quantitatively by the same multiplex system to compare SCYLV-infected versus SCYLV-free plants of the same cultivar and to compare transcripts between SCYLV-resistant and SCYLV-susceptible cultivar.

Material and Methods

Plant material

Cultivars H73-6110, H87-4319 and H87-4094 were obtained from the Hawaii Agriculture Research Center, Aiea, Hawaii, USA. A virus-free line of the cultivar H87-4094 was produced by meristem tip tissue culture and was provided by Dr. A. Lehrer, Honolulu. The plants were grown in the greenhouse at 24°C with a 12-h photoperiod and propagated 1-2 times per year from cuttings.

Isolation of RNA from plant tissues

Plant material (sink leaves, source leaves, mature internodes; #8 and #9) were ground in liquid nitrogen to fine powder and total RNA was extracted using the MagMax 96 total RNA Isolation Kit (Applied Biosystems, Darmstadt, Germany). About 100 mg were transferred to impact-resistent tubes filled with ceramic beads (Precellys Ceramic Kit 1.4 mm; Peqlab Biotechnologie GmbH, Erlangen, Germany) and overlaid with 200 µl lysis/binding solution including Plant RNA isolation aid (Applied Biosystems, Darmstadt, Germany). Tissue samples were homogenized in a FastPrep instrument (MP Biomedicals Europe, Illkirch, France) at a speed setting of 6 m/sec for 40 sec. The tissue homogenate was cleared by centrifugation (1000 x g for 10 min.) and 50 µl of the clear supernatant were used to isolate genomic DNA using the MagMax 96 total RNA Isolation Kit adapted to the KingFisher automated purification system (Thermo Scientific, Langenselbold, Germany; for instrument settings using the MagMax 96 Total RNA Isolation Kit see the corresponding application note available at (www.thermo.com). Total RNA was precipitated with ethanol / sodium chloride. The RNA concentration was determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Langenselbold, Germany). The preparations were made in 3 repetitions, i.e. independent RNA preparations from 3 plants of each cultivar.

GenomeLab eXpress GeXP primer design and RNA quantification

Oligonucleotide primers used for quantitative analysis of SCYLV RNA and selected sugarcane mRNAs were designed using the GenomeLab GeXP eXpress Profiler software (Beckman-Coulter, Krefeld, Germany), using published sequences from GeneBank (GU570004, GU570005, GU570006, GU570007, GU570008 and GU570009), in addition sucrose transporters genes (ShSUT1; AY780256 and ShSUT4; GQ485583) and sucrose phosphate synthase (SPSII; EU269038) and reference genes (25S rRNA; BQ536525 and GAPDH; CA254672) are given in Table 1. These chimeric primers consist of gene-specific sequences and universal primer sequences at each 5’ ends.

Table 1. Primer sequences and expected size of PCR products. The nucleotides in upper case are the universal primer sequences, the nucleotides in lower case are the specific primer sequences.

Gene name	Forward PCR primer (5'-3')	Reverse PCR primer (5'-3')	conc. for RT (nM)	product size (nt)
Sugarcane yellow leaf virus				
ORF0-1	AGGTGACACTATAGAATA atggtgcctattctgctcct	GTACGACTCACTATAGGGA gcttggaacggcatctctta	125	173
ORF2	AGGTGACACTATAGAATA agctcgtcattgatcgtgtg	GTACGACTCACTATAGGGA caggaatttggggtcttcaa	500	249
ORF3-4	AGGTGACACTATAGAATA tgctaggctcgagtctccat	GTACGACTCACTATAGGGA caaacaacaacaggctccaa	500	193
ORF5	AGGTGACACTATAGAATA gataatccggacccaaaggt	GTACGACTCACTATAGGGA gtggaggagcataaatcgga	125	137
Sucrose transporters				
ShSUT1	AGGTGACACTATAGAATA tcccgttcatcctctacgac	GTACGACTCACTATAGGGA atgcgcctactctgacacct	125	151
ShSUT4	AGGTGACACTATAGAATA gctggacttgtatggggtgt	GTACGACTCACTATAGGGA aacgataatgccagtcggag	500	166
Sucrose phosphate synthase				
SPSII	AGGTGACACTATAGAATA taagtggccatcattgcgta	GTACGACTCACTATAGGGA aatacaaaaccaacagcgcc	500	181
Reference genes				
25S rRNA	AGGTGACACTATAGAATA cgtggcctatcgatccttta	GTACGACTCACTATAGGGA aacctgtctcacgacggtct	62.5	263
GAPDH	AGGTGACACTATAGAATA gtggtgccaagaaggatgtt	GTACGACTCACTATAGGGA gttgtgcagctagcattgga	125	158

Multiplex cDNA was synthesized using 50 ng total RNA and a reverse transcriptase reaction (GenomeLab GeXP Start Kit, Beckman-Coulter) containing the gene-specific chimeric reverse primer mix (Table 1). The reverse transcriptase reaction was performed in a thermal cycler with the following program: 48°C for 1 min; 42°C for 60 min, 95°C for 5 min, and hold at 14°C. An aliquot (9.3 µl) of the reverse transcriptase reaction was added to a PCR reaction mix containing the gene-specific forward chimeric primer mix, fluorescently-labelled universal forward primer, unlabelled universal reverse primer, $MgCl_2$, and Thermo-Start DNA polymerase (Beckman-Coulter) according to the GenomeLab GeXP Start Kit instructions. The reactions were

transferred to a thermal cycler and run under the following program: initial polymerase activation at 95°C for 10 minutes, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 70°C for 1 min; after completion of the PCR cycles, the reactions were kept at 4°C.

PCR products were separated by capillary electrophoresis (GenomeLab GeXP Genetic Analysis System, Beckman-Coulter) and quantified using the GenomLab eXpress Profiler software (Beckman-Coulter).

During gene set panel development, attenuation, i.e., dilution of reverse primer concentration for abundant RNA species (25S rRNA, ShSUT1, GAPDH, SCYLV ORF0-1 and ORF5), was necessary as part of the panel optimization (Table 1). The goal of this attenuation was to have the peak signal intensities of abundant RNA species within the mid-level of linearity for accurate quantification of gene-specific PCR products. The rationale of the method is outlined in document no. A54001AB available from Beckman-Coulter.

Statistical analysis

Paired t-tests were performed with SigmaPlot 9.0 (Systat Software Inc., Richmond,USA.

Results

Yield of SCYLV-genome fragments

Sugarcane yellow leaf virus has been isolated from Hawaiian sugarcane cultivars with the purpose to analyse the nucleotide sequence and to reveal the phylogenetic relationship of the Hawaiian strain(s) in the framework of the already known SCYLV strains. Sequence fragments covering together the entire sequence were amplified using primers which bind to so far known conserved nucleotide regions. However, some fragments from some cultivars were either not obtained or obtained in such a small quantity that sequencing could not be performed. Those cases in which successful amplification of all fragments was not achieved comprised preparations from some susceptible and some resistant cultivars (Table 2). Successful amplification of all sequence fragments was obtained from three Hawaiian cultivars, two susceptible (H73-6110 and H87-4094) and one resistant cultivar (H87-4319). Their complete sequence was deposited at NCBI (ElSayed et al. submitted). The question was now, whether indeed different quantities of viral genome parts existed in the plant or whether small sequence differences of viral strains, which prevail in certain cultivars, caused insufficient primer binding and poor amplification.

Table 2 Yield of SCYLV-fragments from RNA preparations from source leaf of different sugarcane cultivars.
+ indicates that sufficient amplificate for sequencing was obtained, - indicates that the amplificate quantity was insufficient. The cultivar H87-4094 was in 2 lines, one SCYLV-infected (vinf), the other virus-free (vf). Some cultivars were classified as SCYLV-susceptible (Susc.), others as SCYLV-resistant (Resist.). The H-cultivars were from Hawaii, the C-, CP- and JA-cultivars were obtained as seed pieces from Cuba.

cultivar	Fragment A (ORF0/1)	Fragment B (ORF2)	FragmentC (ORF2/3)	Fragment YLS(ORF3/4)	Fragment D (ORF 5)
H73-6110(Susc.)	+	+	+	+	+
H87-4319(Resist.)	+	+	+	+	+
H78-4153(Resist.)	-	-	-	+	-
H65-7052(Susc.)	-	-	-	-	-
H78-7750(Resist.)	-	-	-	+	-
H87-4094vinf (Susc.)	+	+	+	+	+
C1051-73	-	-	-	+	-
CP52-43	-	+	-	+	-
JA-605	-	+	-	+	-

Quantitative analysis of SCYLV-sequence fragments from 3 Hawaiian cultivars

SCYLV was prepared from sink leaves, source leaves and mature internodes (#8-9) of those three cultivars from which the complete SCYLV-sequence had been obtained. The viral strains exhibited small sequence differences and primers were designed for these strains. The transcript quantities were determined by the one-step GeXP Multiplex system where up to 30 transcripts from a particular preparation are measured in parallel. The amplified segments are located in the 6 ORFs of the viral genome (Figure 1). Ribosomal RNA (25S rRNA) was used as an internal control and the transcript quantities were related to this control. RNA-preparations from virus-free plants were included in the analysis as a negative control. GAPDH transcripts were also included as another housekeeping control.

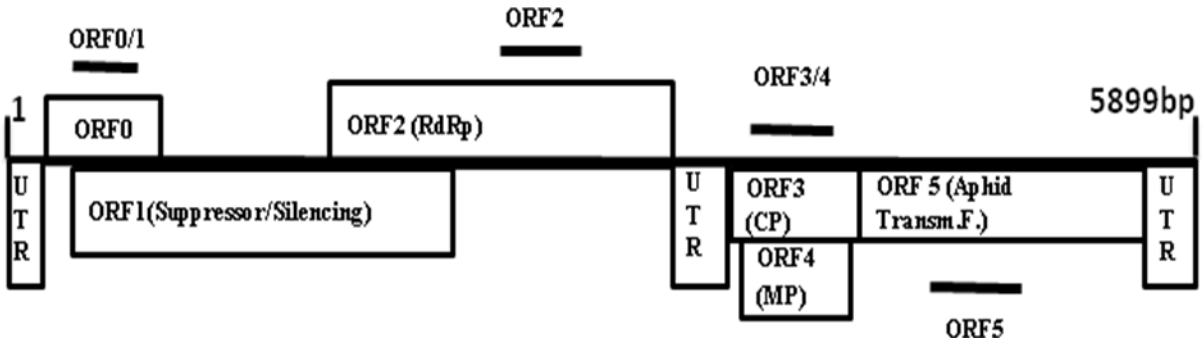

Fig.1 Organization of the SCYLV genome (after Moonan et al. 2000, modified). The position of the amplified sequences is indicated.

The ORFs seem to be present in very different quantities in the RNA preparations, there were differences between the cultivars and differences between the plant organs from where the preparations had been obtained (Figure 2). The primer concentrations were attenuated to yield clear signal strengths (Table 1), which has to be considered when direct quantitative comparison of different amplificates is attempted. Sink leaves contained twice or more SCYLV-transcripts than source leaves and source leaves contained more SCYLV-transcripts than mature internodes, all values related to 25S rRNA. As large as the differences between the plant organs were the differences between the genome parts. The fragment containing ORF0-1 (which codes for a suppressor/silencer gene) was highly expressed in all cases and of at least twice the quantities of any other fragment in sink and source leaves, but not so in internodes. The fragment containing ORF2 (coding for RNA-dependent RNA polymerase) appears second or third highest together with ORF5 (which codes for an aphid transmission factor) in sink and source leaves and at same quantity as ORF0-1 in internodes, however the primer concentration for ORF2 was three times that of ORF0-1 or ORF5 (Table 1). The fragment coding for ORF3-4 (which codes for the capsid protein) was the lowest in all cases. The preparation from virus-free plants cv. H87-4094vf did expectedly not give a significant amplificate, except of a trace of ORF0-1 in sink leaves (1% of the value of the infected cultivar).

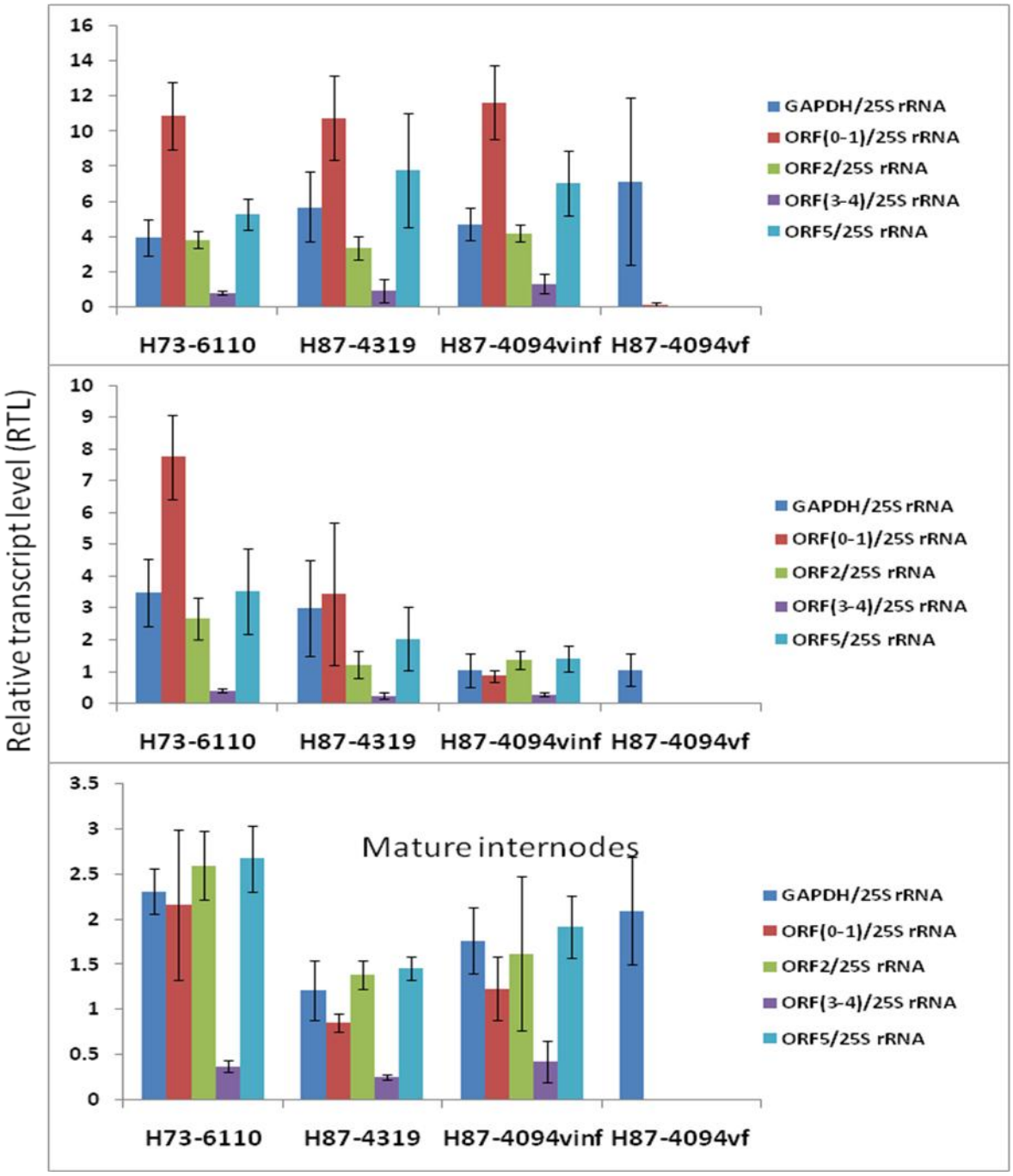

Fig.2 Relative transcript levels (RTL) of four different fragments of the SCYLV-genome covering ORFs 0 to 6 from four sugarcane cultivars respectively lines, including RTL of GAPDH as housekeeping enzyme. (**a)** RNA-preparation from sink leaf, (**b**) from source leaf, (**c**) from mature internode (#8 and 9).The transcript levels of the SCYLV-fragments and of GAPDH were related to the transcript level of 25S rRNA of the particular RNA-preparation. Mean and SD,3 repetitions.

The transcript levels were the same in the sink leaves of the 3 infected cultivars, whereas the transcript levels in source leaves and internodes from cv. H73-6110 were twice of those from H87-4094 and H87-4319 (Figure 2). It was surprising that H87-4319 which had been classified as SCYLV-resistant because of its negative response to tissue blot immunoassay (Schenck & Lehrer, 2000) had as high transcript levels as the susceptible cultivars. The sum of the relative transcript levels was calculated as a measure for virus titre showing the different values of viral transcripts in sugarcane cultivars and their organs (Table 3); the values are dominated by the transcripts ORF0-1 and ORF5 which were amplified with the same, low primer concentrations, therefore no correction was attempted for the less amplified ORFs 2 and 3-4. The different transcript levels for the ORFs are evident when the ratios between the amplificates were calculated for each cultivar and each plant organ.

Table 3 Sum of ORF transcript levels as average measure of virus titre. The relative transcript levels (RTL) of the 4 SCYLV-fragments were added up for each cultivar and plant organ. No correction was made to account for the different primer concentrations (Table 1).

SCYLV from cultivar	**plant organ**	**Sum of relative transcript levels (ORF0-5)/25S rRNA**
H73-6110 (susc.)	Sink leaf	20.8
	Source leaf	14.3
	internode	7.9
H87-4319 (resist.)	Sink leaf	23
	Source leaf	6.9
	internode	4.1
H87-4094 (susc.)	Sink leaf	24.3
	Source leaf	4
	internode	5.1
H87-4094 virus-free	Sink leaf	0.1
	Source leaf	0
	internode	0

There were different ratios between the ORF-transcripts in sink leaves and internodes, and also different ratios between some cultivars (Table 4). The largest variations were seen in the ratio of ORF0-1 to ORF2, especially in the source leaves. Although the primers for the four sequence parts had been designed based on the nucleotide sequences of the SCYLV-strains from the three Hawaiian cultivars, it may be speculated that three-dimensional conformations of the different

parts of the viral genome as well as slightly different primer binding kinetics caused a differently efficient amplification of sequence parts in the Multiplex system. If that is the case, then the apparent transcript levels which were obtained by this method should be at a fixed ratio from wherever the RNA-preparation was derived from. The average relative transcript level was calculated for each sequence fragment and then the actual relative transcript level for each cultivar was drawn (Figure 3). If there is a constant ratio of fragment transcript levels then the graph should be parallel to the x-axis, i. e. a fixed proportion of expressions of the ORFs. This is indeed the case for the preparations from sink leaves and from internodes, however there is significant deviation from a fixed transcript ratio in source leaves (Figure 3), i. e. there is far above average expression of ORF0-1 in H73-6110 and far below average expression of ORF0-1 and ORF5 in H87-4094. These differences were significant despite the low number of repetitions (Table 5).

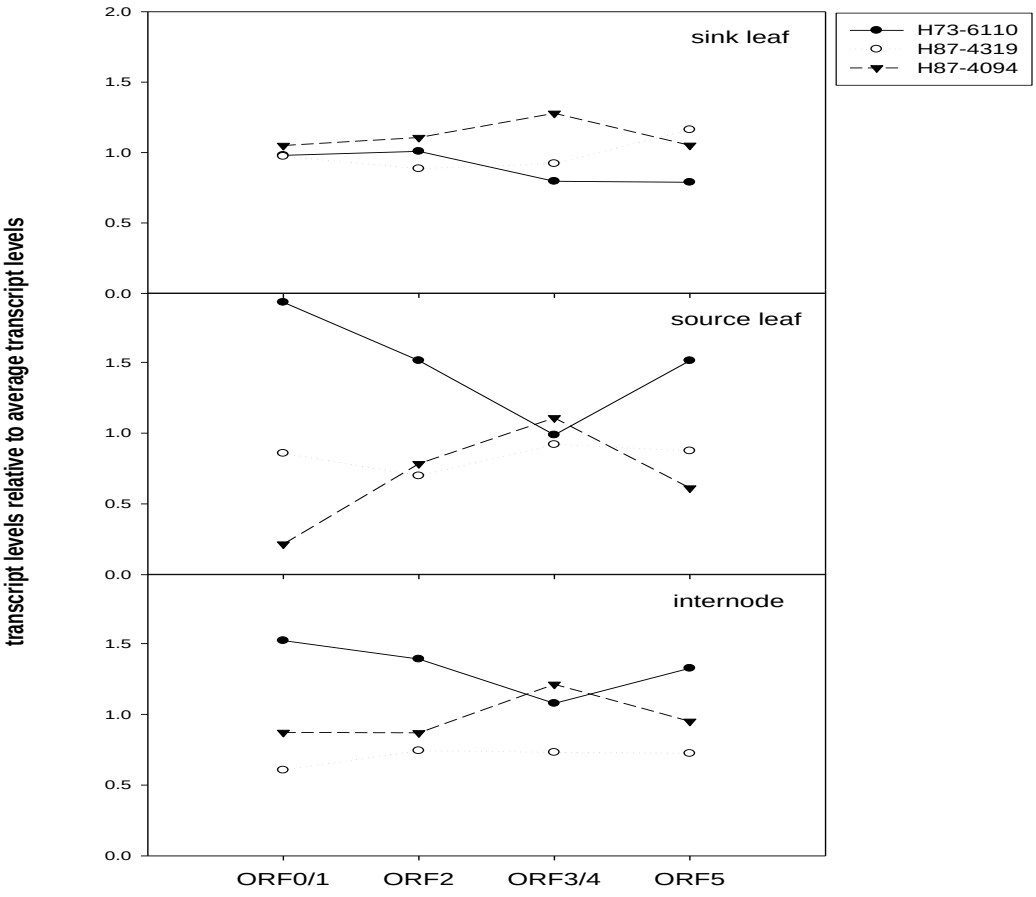

Fig. 3 Relative transcript levels (RTL) of the ORFs (ORF/25S rRNA) compared to the average RTLs of the ORFs in the organs of the 3 infected cultivars. The average for each RTL of the ORFs from the 3 cultivars H73-6110, H87-4319 and H87-4094 was formed for sink leaf, source leaf and internodes. Then the actual transcript level of each cultivar in sink, source and internodes was calculated relative to this average value. Stars indicate that the value is significantly different from the neighbouring value of the same cultivar (light star indicates a trend ($P<0.1$), full star (*) indicates significane ($P<0.05$) and double star (**) high significance ($P<0.01$)).

Table 4 Ratios of relative transcript levels (normalized with 25S rRNA) of SCYLV-fragments from the 3 cultivars (not taking in account the attenuated primer concentrations).

SCYLV ORFs	Sink leaf	Source leaf	Mature internode
ORF0-1 / ORF2			
H73-6110	2.84	2.92	0.83
H87-4319	3.20	2.81	0.62
H87-4094	2.76	0.63	0.76
ORF0-1 / ORF3-4			
H73-6110	13.2	28.7	5.68
H87-4319	11.3	13.8	3.31
H87-4094	8.80	2.9	2.95
ORF0-1 / ORF5			
H73-6110	2.05	2.20	0.81
H87-4319	1.38	1.70	0.59
H87-4094	1.65	0.61	0.65

Table 5 Significance of differences in transcript pattern between different genome fragments (ORFs) from source leaf preparations. The ratios were not corrected for the attenuated primer concentrations (Table 1). Paired t-test was applied to the results for source leaves of Fig. 2 and Table 4.

Source leaves	Paired t-test	P value	Significance
cv. H73-6110	ORF0/1 × ORF2	0.066	trend
	ORF0/1 × ORF3/4	0.10	trend
	ORF3/4 × ORF5	0.090	trend
cv H87-4094	ORF0/1 × ORF2	0.014	significant
	ORF0/1 × ORF3/4	0.012	significant
	ORF0/1 × ORF5	0.032	significant
cv. H87-4319	ORF2 × ORF3/4	0.031	significant
	ORF2 × ORF5	0.012	significant
	ORF3/4 × ORF5	0.006	highly significant

Transcript levels of sucrose-storage related genes in sugarcane

Previous analysis had shown that SCYLV-infected plants were probably hindered in sugar export from source leaves leading to carbohydrate backup in source leaves (Lehrer et al. 2007 and Yan et al. 2009) and stunted growth of internodes (Lehrer & Komor, 2008). The multiplex analysis was used to quantify the transcript levels for sucrose synthesis (SPSII) and sucrose transporters (ShSUT1 and ShSUT4) in parallel with the viral transcript levels in the same RNA preparations. Also included was GAPDH, which had been used previously as a standard for house-keeping transcript, because the expression levels of 25S rRNA and GAPDH were higher and more consistent across sugarcane tissues than β-actin and β-tubulin (Iskander et al. 2004). GAPDH-transcripts were not constant relative to the 25S rRNA transcripts. The values were higher in sink leaves than in source leaves and especially in internodes. Furthermore there were differences between cultivars, especially the values for cv. H87-4094 (both, infected and virus-free) in source leaves were much lower than in H73-6110 and H87-4319. Thus there was no indication that GAPDH is a better internal standard than 25S rRNA in sugarcane.

Sucrose-phosphate synthase (SPSII) transcript levels were approximately the same in sink leaves, source leaves and internodes (Figure 4), it varied less in plant organs than GAPDH and SCYLV-transcripts. SPSII was chosen because it is a key enzyme in sucrose synthesis in leaves and sucrose storage in internodes (Huber and Huber, 1996; Komor et al. 1996; Zhu et al. 1997 and Casu et al. 2003). Two types of sucrose transporter were found as transcripts, one belonging to the SUT1-group (Casu et al. 2003) and the other belonging to the SUT4-group. The latter had not been described so far for sugarcane besides the publicly available NCBI Genbank entry, but was clearly identified by alignment (Figure 5). Both transporter transcripts were measured. ShSUT1 was highly transcribed in sink leaves (RTL 3-6), less so in source leaves (RTL 1-2) and internodes (RTL 0.3-0.8) (Figure 4). ShSUT4 exhibited a constant transcript level in sink leaves, source leaves and internodes, very much in contrast to ShSUT1, therefore the ratio of the amplificates of ShSUT1/ShSUT4 changed strongly from above 6 to below 1 (Table 6).

There were no obvious differences in transcript quantities for sucrose transporters and SPSII in sink leaves and source leaves between susceptible cultivars (H73-6110 and H87-4094) and the resistant (H87-4319) or virus-free cultivar (H87-4094vf). The resistant cultivar had however a significantly higher transcript level of ShSUT1 than the two susceptible cultivars (paired t-test: P=0.02). The comparison of the virus-free with the infected plants of cv. H87-4094 gave the impression that the infected plants had less GAPDH and ShSUT1 transcripts, however the differences were insignificant (paired t-test: P=0.2-0.5).

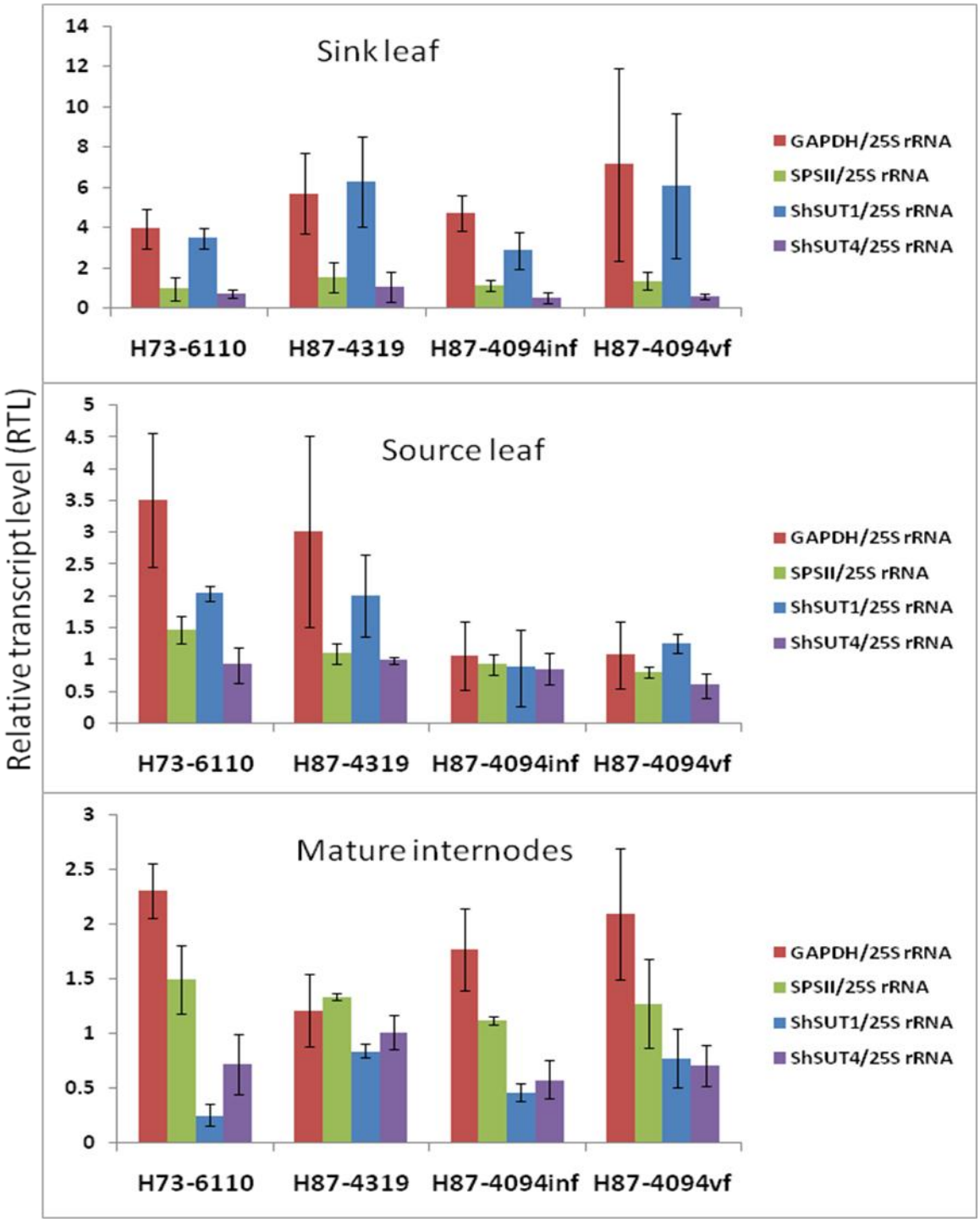

Fig. 4 Relative transcript levels (RTL) of GAPDH, SPSII, ShSUT1 and ShSUT4 from four sugarcane cultivars respectively lines. (**a)** RNA-preparation from sink leaf, (**b**) from source leaf, (**c**) from mature internode (#8 and 9). The transcript levels were related to the transcript level of 25S rRNA of the particular RNA-preparation. Mean and SD, 3 repetitions.

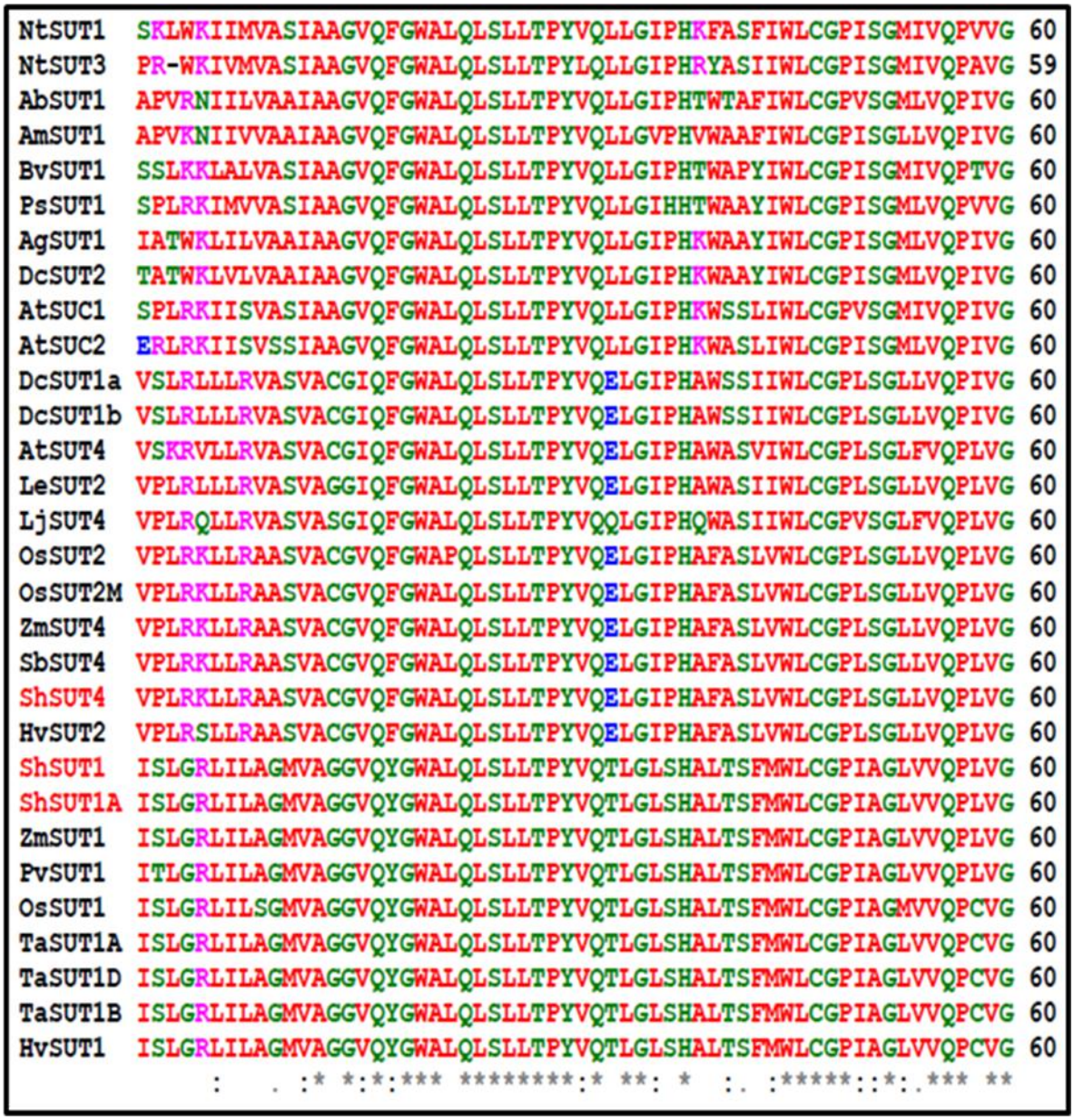

Fig. 5a Alignment of the amino acid sequences of sugarcane sucrose transporters ShSUT1 and ShSUT4 (in red) to published sucrose transporter sequences from monocots Asterisks indicate perfect matches within all sequences. The sequences were aligned using CLUSTAL W.

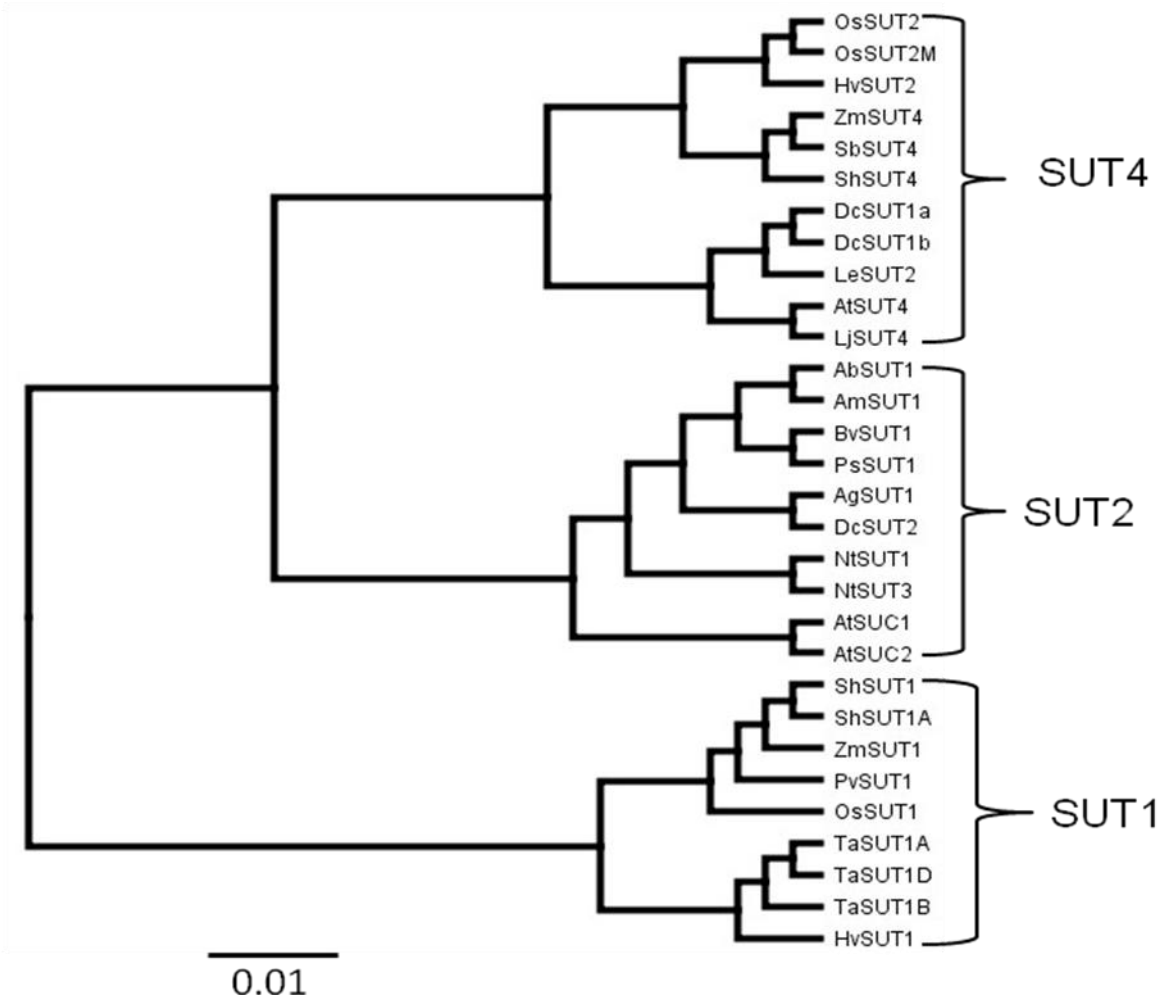

Fig.5b Phylogenetic relationships. The selected sucrose transporters from monocots were ShSUT4 (*Saccharum hybrid* sucrose transporter, GenBank accession number ACV95498, SbSUT4 (*Sorghum bicolor*; ACX71839), ZmSUT4 (*Zea mays*; AAT51689), OsSUT2M and OsSUT2 (*Oryza sativa*; AAY83288; BAC67163 respectively), HvSUT2 (*Hordeum vulgare*; CAB75881), AbSUT1 (*Asarina barclaiana;* AF191024), AgSUT1 (*Apium graveolens*; AF063400), AmSUT1 (*Alonsona meridionals*; AF191025), AtSUC1 (*Arabidopsis thaliana*; X75365), AtSUC2 (*Arabidopsis thaliana*; X75382), AtSUT4 (Arabidopsis thaliana; AF175321), BvSUT1 (*Beta vulgaris*; U64967), DcSUT1a (*Daucus carota*; Y16766), DcSUT1b (*Daucus carota*; Y16767), DcSUT2 (*Daucus carota*; Y16768), HvSUT1 (*Hordeum vulgare*; AJ272309), LeSUT2 (*Lycopersicon esculentum*; AF166498), LjSUT4 (*Lotus japonicas*; AJ538041), NtSUT1 (*Nicotiana tabacum*; X82276), NtSUT3 (*Nicotiana tabacum*; AF149981), OsSUT1 (*Oryza sativa*; D87819), , PsSUT1 (*Pisum sativum*; AF109922),PvSUT1 (*Panicum virgatum*; FJ839440), ShSUT1 (*Saccharum* hybrid; AY780256), ShSUT1A (*Saccharum hybrid*; GU812864), TaSUT1A (*Triticum aestivum*; AF408842), TaSUT1B (*Triticum aestivum*; AF408843), TaSUT1D (*Triticum aestivum*; AF408844) ZmSUT1 (*Zea mays*; AB008464). The

phylogenetic tree is based on the alignment of amino acid sequences. and was constructed with Geneious program and UPGMA method, the scale bar represents the genetic distance.

Table 6 Ratio of SUT1 to SUT4 in the cultivars and plant organs. The attenuated primer concentrations (Table 1) were not taken into account. Paired t-test was applied to the data to show the significance of differences, t= trend with P<0.1, *= significant with P<0.05, the small letters indicate which data pairs are significant, e. g. the data with a are not significantly different to each other, but significantly different to data with b or c.

Cultivar	**Organ**	**ShSUT1/ShSUT4**	**Significance**
H73-6110	Sink	4.8±1.4	***a**
	Source	2.3±0.6	***b**
	Internode	0.39±0.25	***c**
H87-4319	Sink	6.7±2.0	***a**
	Source	2.0±0.6	***b,+c**
	Internode	0.84±0.17	***+c***
H87-4094 inf	Sink	6.6±3.6	***+a***
	Source	0.93±0.52	***+b***
	Internode	0.81±0.11	***+b***
H87-4094 vf	Sink	6.2±0.6	****a***
	Source	2.3±0.7	****b,+c***
	Internode	1.09±0.09	***+c***

Discussion

The transcript levels of the viral genome were very different depending on which part of the genome was considered. The differences are apparently not a methodological artefact of the amplification process, since the ratio of transcripts was not constant but depended on plant organ and plant cultivar from where the viral preparation had been obtained from. The transcript levels (relative to 25S rRNA transcripts) of a particular gene can be quantitatively compared between different preparations, organs or cultivars. The comparison of amplificates of different genes is

complicated by the different, attenuated primer concentrations (Table 1) and possibly by different primer binding efficiencies. The ratios of amplificates (e. g. in Tables 4-6) are still a valid information showing whether differences are significant or not, but they may not represent exactly the ratio of templates because of different primer concentrations. Data obtained with the same primer concentrations (e. g. ORF0-1, ORF5 and ShSUT1) may be more likely representative for the real ratio of templates in the RNA-preparation.

ORF0-1, which codes for a silencer/suppressor (Figure 1), was always the highest or among the highest transcribed viral region and it is understandably of great importance for the viral proliferation in the plant. ORF0 is expressed early during infection by *Potato leaf roll virus* and was found to be responsible for symptom development (Van der Wilk et al. 1997). In addition, the ORF0 protein may be involved in an interaction with a host specificity factor (Sadowy et al. 2001).The next ORF of SCYLV, ORF2 is less often transcribed and ORF3-4 even less. Possibly the transcription of the viral genome by the RNA-dependent RNA-polymerase becomes less efficient along the length of the viral RNA and falls off more frequently the longer it travels along the viral genome. Amazingly then that transcripts of ORF5, the last ORF of the genome, are much more frequent than transcripts of ORF3-4. Thus, either there is (or are) more initiation sequences for the RNA-dependent RNA-polymerase, not only at the 5´-end of the genome, or there are RNAses in the plant which selectively degrade parts of the viral transcripts. RdRp can probably also produce transcription variation by replication slippage, which results from short repeats. Although no direct evidence for this slippage exists, the presence of short repeats in some viral genes may suggest it (Hancock et al. 1995). The amplification of ORF0-1 and ORF5 were achieved with one fourth of the primer concentration necessary for ORFs 2 and 3-4. Therefore the differences observed, namely that ORF0-1 and ORF5 appear higher expressed than ORF2 and especially ORF3-4 is expected to be even larger in the samples than it appears in Figure 2.

The transcript levels of ORF2 varied between cultivars and plant tissues (sink, source, internodal tissues). ORF2 codes for the RNA-dependent RNA polymerase (RdRp) of SCYLV (Figure 1) and therefore directly determines the SCYLV-titre in the tissue. The ORF3 of SCYLV contains also ORF4, but in a different frame (Smith et al. 2000), and thus codes for two proteins, the capsid protein and a “genome–linked viral protein (VPg)”. This ORF3-4 has the lowest transcript levels, which was unexpected, since it is considered as a diagnostic region for SCYLV. It indeed exhibits the lowest diversity among the SCYLV-strains, only half of the diversity compared with other genome parts (ElSayed et al. submitted), which may explain why it is more likely amplified by conserved primers than other ORFs (Table 1), despite its low transcript levels. ORF5 is produced by translational read-through of a (UAG) amber stop codon of ORF3 and

codes for a putative "aphid transmission factor". The transcript levels of ORF5 were always high in all plant stages (Figure 2), which is obviously very important for the infection and propagation of the virus from plant to plant. The very different transcript levels of the 6 ORFs makes it difficult to quantify exactly the virus titre of plants or tissues, since it will depend which ORF is selected for amplification by real-time RT-PCR. Thus the term virus titre may be ambiguous, whether it means the titre of the best expressed ORF or the sum of expressed ORFs (Table 3).

The viral transcript levels were not only different with regard to the different ORFs but also with regard to the plant tissue from where the RNA was prepared from. SCYLV is confined to the phloem (Vega et al. 1997 and Yan et al. 2009) and a simple explanation would be that sink leaves have a relatively higher phloem content than source leaves and these a higher phloem content than internode tissues. There are no data on quantitative determination of phloem-located RNA in sink, source and internodes, but the visual microscopic impression from leaves and internodes of sugarcane does not support that conclusion. It appears more likely that viral replication is more "successful" in sink leaves than in other tissues and least in mature internodes.

The transcript levels of plant genes which are involved in sucrose storage, namely SPS and sucrose transporters, were determined from the same RNA-preparations as the viral ORFs. SPS had been found previously to be of decisive importance together with vacuolar acid invertase for sucrose storage in sugarcane internodes (Zhu et al. 1997). The primers for sugarcane SPS were designed from conserved regions after alignment of published sequences (not shown). SPS transcripts were present at approximately the same concentration in all tissues and appear to be less expressed than SUT1 in sink and source leaves, although the primer concentration for SPS was fourfold of that for SUT1. The ratio of SUT1/SPS decreased in mature internodes because they had relatively low SUT1-levels. SUT4, which had not been described so far in sugarcane, is at constantly low transcript levels in all tissues. Because of the decreased level of SUT1 in internodes, SUT4 becomes more important in this storage tissue (but it was amplified with fourfold higher primer concentration than SUT1). Labelling experiments had previously shown that the cycling of sucrose through synthesis and hydrolysis (futile cycle) decreases during maturation of the internodes together with a stronger contribution of sucrose uptake at the expense of hexose uptake (Komor et al. 1996). Thus sucrose synthesis appears at first glance to proceed at similar rates in the three tissue types and at a rate smaller than sucrose transport. However it has to be kept in mind that transcript levels may not be directly correlated with enzyme activities, especially not in a highly regulated enzyme such as SPS (Huber and Huber, 1996).

No significant differences in transcript levels were observed between the SCYLV-infected and the virus-free line of cultivar H87-4094, except of course when the viral ORF-transcripts were considered. The comparison of transcript levels in the two susceptible cultivars with those in the resistant cultivar did not allow a reliable conclusion. Although the pattern of transcript levels (GAPDH, SPS and SUTs) is different between the resistant and the susceptible cultivars in internodes (Figure 4), the two susceptible cultivars show a strong difference between themselves in source leaves (Figure 4). Thus obviously cultivar differences are overriding possible differences in SCYLV-susceptibility. Reliable conclusions may be drawn only when a large number of susceptible and resistant cultivars would be compared. The large genome of sugarcane and the accompanied large redundancy of genes (Ming et al. 1998) may cause a large cultivar-specific variation of gene expression, leading to virus resistance by several unrelated paths in a regulatory network. Besides that the term SCYLV-resistance in sugarcane cultivars has become questionable in front of the relatively high transcript levels of SCYLV-ORFs in the "resistant" cultivar H87-4319. Possibly there are clone-specific differences or even a mixed infection in some clones with more than one SCYLV and with variable transcription efficiencies. When the original screenings of cultivars for SCYLV were performed using tissue blot immunoassay (Schenck and Lehrer, 2000), 3 out of 244 tested plants tested positive for SCYLV. The few positives were not taken serious and blamed on possible methodological mistakes. But it may be that these positives were due to clonal variation or happened to stem from viral titre fluctuations, which were found to be very strong in one commercial cultivar (H73-7052, Lehrer & Komor, 2008). Future experiments need to concentrate on clonal variations of sugarcane cultivars and mixed infections together with different virulence of SCYLV-strains in a cultivar as possibilities to explain so far inconsistent observations.

Acknowledgements

This study was funded by the Bayreuth University. We thank Michaela Hochholzer for excellent technical assistance. The help and advice by members of the plant physiology institute (University Bayreuth, Germany) is gratefully acknowledged. A. Elsayed is grateful to the Egyptian Government for the scholarship.

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9. List of publications:

The following papers have been published / submitted / to be submitted during the work on this thesis:

1. Axel Lehrer, Shih-Long Yan, Blanca Fontaniella, **Abdelaleim Elsayed**, Ewald Komor, *Journal General Plant Pathology*, **2010**, 76: 62-68.“ Carbohydrate composition of sugarcane cultivars that are resistant or susceptible to Sugarcane yellow leaf virus”.

2. Ewald Komor, **Abdelaleim Elsayed**, Axel Lehrer, *European Journal Plant Pathol*ogy, **2010**, 127, 207-217.“ *Sugarcane yellow leaf virus* introduction and spread in Hawaiian sugarcane industry: Retrospective epidemiological study of an unnoticed, mostly asymptomatic plant disease”.

3. **Abdelaleim Elsayed**, Alfons Weig, Ewald Komor, *European Journal Plant Pathol*ogy (submitted)“ Molecular characterization of Hawaiian Sugarcane yellow leaf virus genotypes and their phylogenetic relationship to strains from other countries”.

4. **Abdelaleim Elsayed**, Mohamed F. Ramadan, Ewald Komor, *Physiological and Molecular Plant Pathology* (submitted) “Expression of sucrose transporter (ShSUT1) in a Hawaiian sugarcane cultivar infected with Sugarcane yellow leaf virus (SCYLV)”.

5. **Abdelaleim Elsayed**, Alfons Weig, Ewald Komor, *Plant pathology* (submitted) “Simultaneous quantitative analysis of transcripts for Sugarcane yellow leaf virus, sucrose transporters and sucrose phosphate synthase in Hawaiian sugarcane cultivars by multiplex RT-PCR”.

6. **Abdelaleim Elsayed**, Ewald Komor, *Archives of Virology* (to be submitted) “Sequence deletion in sugarcane yellow leaf virus genome and their effect on the diversity of virus population”.

ACKNOWLEDGMENTS

In the name of Allah, The Most Beneficent, the Most Merciful. I thank Allah for giving me power to accomplish this PhD-project. I thank my family for the unconditional support and encouragement during all the steps of the program. I thank the Egyptian Government, Ministry of Higher Education, for sponsoring of my graduate studies abroad.

I want to express my gratitude to Prof. Dr. Ewald Komor, my supervisor, for his guidance, patience and encouragement. I am thankful to him for the liberty and flexibility he gave me during the time I spent in his Department. Without all these favors from him, accomplishment of this work would have been extremely difficult. Many thanks also to Bayreuth University, Prof. Dr. Stephen Clemens and to Prof. Dr. Ortwin Meyer, for their kind assistance to find financial support to achieve and complete this work. I am very grateful to Dr. Alfons Weig for guiding me in the field of molecular biology. During my academic and professional carrier I benefited from the assistance of many people. I especially thank Dr. Sabastian Fettig, Dr. Aleksandra Trampczynska, Dr. Katja Hartig, Dr. Michael Weber, Dr. Paul Ziegler, Mrs. Susanne Bauer, Mr. Ulrich Deinlein, Mr. Daniel Peisker, Mr. Stefan Rensch, Mr. Tran Dang Khoa, Mr. Daniel Eduardo Souza-Canada, Mr. Deepak Pant and technical assistances; Mrs. Pia Schuster and Christiane Meinen for the limitless help and friendship.

I am deeply thankful to Prof.Dr. Thomas Laux, Freiburg University and Mr. Steffen Knauer for providing the chemicals and devisors and helpful to achieve the *In Situ* hybridization experiment.

I would like to thank to my friends for their support and encouragement in different ways: Dr. Mohamed Ali, Dr. Salah Khalifa, Dr. Mohamed Abdelhamid, Prof. Dr. Kamel Shaker, Mr. Ramzi Ben Amara, Mr. Amr Ismail, and Dr. Ilyes-Aissa Chahma.

Special thanks to my friend Dr. Mostafa Abdelkhalik for his brilliant advice and encouragement and to Dr. Tarek Hewazi for the useful discussions and for critical reading of my manuscript.

I am deeply thankful to Mrs. Ursula Ferrera the secretary of Department of Plant Physiology for her kind assistance and support.

Dr. Mahmoud Dohiem, Dr. Mohamed Fawzy, Dr. Ahmed Omar and Dr. Mamdoh Kamel taught me the fundamentals of biochemistry and plant molecular biology in Egypt. I really appreciate their guidance and I am thankful to them for sharing their knowledge and experience. Last but not least, special gratitude and thanks to my wife, Faten, for her immeasurable love, constant and unconditional patience, and encouragement.

Declaration / Erklärung

Hereby I declare that this work has so far neither been submitted to the Faculty of Biology, Chemistry and Earth Science at the University of Bayreuth nor to any scientific institution for the purpose of doctorate. Furthermore, I declare that I have written this work by myself and that I have not used any other sources, other than mentioned earlier in this work.

Hiermit erkläre ich, dass diese Arbeit von mir weder an der Fakultät für Biologie, Chemie and Geowissenschaften der Universität Bayreuth noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde. Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel benutzt habe.

Bayreuth, den

Abdelaleim Elsayed